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ACCESSORY GROWTH FACTORS FOR BACTERIA AND RELATED MICROORGANISMS

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CONTENTS

A number of studies in bacterial nutrition have dealt with the so-called growth factors, accessory substances, vitamins, or growth activators,[1] substances which in small amount appear to play an important part in the development of certain bacteria

[1] The term hormone has also been used occasionally to designate these substances, but it does not seem to be valid on the basis of usage in mammalian and plant physiology

and allied forms The nature of these substances together with their possible function in the metabolic activity of various microorganisms has been a matter of conjecture for some time Recently some interesting results have been secured which should lead to a much better understanding of the entire subject

In the past some confusion has been caused by a rather indiscriminate application of similar terms to a variety of effects In some instances the effect of an added substance has been merely that of stimulation, resulting in more abundant or more rapid development in an environment in which cell proliferation was already taking place This stimulation has been due, at times, simply to adding more available food material to a "starvation" medium In other cases, however, the added material has seemed to play a more important part in that its presence appeared to be necessary for development At times very small amounts of added substance have permitted good growth in a medium in which the organism was not ordinarily able to multiply

Substances which exert one or another of these effects have been found in a wide variety of extracts of plant and animal tissues Through fractionation of these extracts attempts have been made to obtain the active components in pure form and to learn something of their chemical structure The observations have been scattered over a rather wide field dealing with many miscellaneous sources of growth factors and with microorganisms of quite diverse groups Before reviewing this material in more detail it seems best to consider briefly the basic ideas which have guided the investigational work

It has usually been assumed that unknown chemical entities of organic nature were supplied by the added extract, and that these hypothetical substances produced the growth-promoting effect Other interpretations have been advanced from time to time These can be summarized as follows (1) The added material may have supplied certain needed inorganic salts, particularly those of metals which act as catalysts, (2) the added material may have combined with and removed from activity

an excess of some constituent which was present originally in sufficient concentration to be toxic, or (3) it may have altered the physical characteristics of the medium so that cell proliferation became possible Sole reliance on any of these explanations makes unnecessary the assumption of the existence of essential accessory substances of organic nature

At the present time we appear to have reached a point from which a clearer idea of these effects may be gained In a few instances it has been found possible to substitute, either fully or in part, known chemical compounds for the indefinite growth-promoting entities of tissue extracts Eventually we should be able to abandon the use of such terms as V and X factors, L fractions, and others of like nature as their specific identities become established

It seems best to review first the fractionation of tissue extracts and other sources, pointing out those instances where the active substance has been identified or where a knowledge of its properties has progressed to such a point that its nature could be surmised and a known compound substituted successfully for it A consideration of the other explanations of growth-factor activity will follow No attempt will be made to review the earlier work, other than that needed as a background for the present discussion, since former studies have been summarized by Knorr (56), Sergeant (142), Peskett (111) and Knight (52) Also, no attempt will be made to treat the subject of plant auxins Various bacteria and other fungi are important in the production of substances, such as 3-indoleacetic acid, which exert a marked influence on plant cell elongation and multiplication So far as the writers are aware, however, there is no instance on record as yet in which auxin-*a*, auxin-*b*, 3-indoleacetic acid or related compounds have proved to be essential for cell proliferation of bacteria The production of these substances by bacteria and their effects on plants is outside the scope of this article and readers are referred to the recent publications of Boysen-Jensen (6), Schlenker and Rosenthal (128), Went and Thimann (164), Nicol (101) and others

EXPERIMENTAL PROCEDURES

In experimental work dealing with the growth accessory factors for microorganisms details of technique are quite important Although these items have been mentioned from time to time in studies on bacterial metabolism, often they have been neglected, and so it seems desirable to emphasize again certain points

Sterilization of the fractions to be tested for growth-factor activity presents a problem at the outset The unknown substances in tissue extracts may be destroyed by heat, while resort to filtration may cause serious loss due to inactivation or adsorption The checking of one method against the other is useful, though there is the possibility that some of the factors may be inactivated by both procedures

Basic medium for the tests In tests for growth accessory factors, the basic medium should be adequate in all other respects and the conditions of cultivation should approach as closely as possible those known to be most suitable for the organism in question Unfortunately our knowledge of these requirements is notoriously inadequate Concerning many microorganisms little is known of their actual needs with respect to various amino acids and other nitrogenous ingredients, the inorganic salts and their proper physiological balance, and other factors such as osmotic pressure, surface and interfacial tension, gaseous environment, redox potential and kindred conditions Consequently our efforts are often seriously hampered at the start, and it is uncertain whether these important conditions are being satisfied To avoid the uncertainties regarding amino acid requirements, some investigators have used hydrolyzed casein or gelatin as a basic medium This procedure, however, is subject to the disadvantage of introducing impurities with the casein and gelatin, and also in that the medium is no longer of known composition

Size of inoculum Inoculation of the test medium with large numbers of cells may introduce appreciable amounts of growth factors, either from the cells themselves or from the previous culture medium While this added material may be eliminated by several successive transplants in the new environment, in

general it would appear to be more desirable to start with smaller numbers of cells, thus affording at the outset a stricter test of the ability of the cells to multiply in the new medium

Storage of growth factors in cells If cultures are grown in a medium containing an excess of some required factor, they may possibly store sufficient quantities of the material to permit of further limited proliferation when transferred to a deficient medium This could well lead to erroneous conclusions or at least make difficult the interpretation of results

Multiple growth requirements Tissue extracts contain many biologically active substances Liver, for example, has been shown to contain a number of the accessory substances as well as amino acids Therefore in attempting to isolate growth substances for microorganisms due consideration must be given the multiplicity of possible factors This is well exemplified in the work on bios It must be borne in mind too that amino acids frequently accompany accessory factors through the earlier stages of attempted separation If one or more of these amino acids is essential for growth and if it is not supplied in the basic medium, its subsequent removal during chemical manipulation will give rise to a deficient environment and the organism under investigation will be unable to multiply for this reason rather than because of a lack of other needed substances

Methods of assay Of the several methods which have been proposed for the quantitative determination of the potency of growth-factor preparations, by far the most widely used is that of visual inspection of the culture tubes for turbidity This method has a large error but is simple and rapid The use of a cell and thermocouple for the determination of the density of the suspension probably increases the accuracy of this method (169) More precise results can be obtained by direct count but where a large number of determinations is to be made, the time required would be a serious disadvantage Direct weighing of the mass of organisms formed has been employed In the case of the mycelium of molds (16) weighing probably gives accurate results, but with bacteria and yeasts it is doubtful whether the method is much more accurate than visual inspection Indirect

determination (92) of the mass of bacterial growth by means of its nitrogen content is probably more accurate than inspection, although subject to some rather serious errors due to the possibility that nitrogenous material from the medium might be included with the organisms, or if the organisms are washed too thoroughly nitrogen may be lost Sternfeld, Wermuth and Saunders (148) attempted to follow growth by means of changes in conductivity, refractive index and other physical properties of the cultures, but the differences were too slight to be useful Some workers (50, 144) have used the titer of acid formed by bacteria as an index of growth

In the following sections discussion of the growth-promoting materials for bacteria and the related non-chlorophyll bearing fungi has been arranged under the different groups of microorganisms

VARIOUS GROUPS OF THE BACTERIA

The streptococci and allied coccus types

The nutritive requirements of the pathogenic streptococci have always been quite obscure These organisms practically without exception fail to develop in various amino acid synthetic media (40, 64, 67, 29) and attempts at separation of essential growth substances from meat infusions or other similar sources seem to have been attended by unusual difficulties In a few instances the active material has been carried through several preliminary stages in the process of purification but little success has been attained beyond this point

By adsorption with fuller's earth and charcoal, Freedman and Funk (37) obtained from beef infusion, autolyzed brewer's yeast and peptone, substances which showed growth-stimulating activity for hemolytic streptococci Substances with a similar growth-stimulating effect were also found on hydrolysis of certain proteins, particularly casein, commercial gelatin, yeast protein and edestin (37) The evidence indicated that the active substances were not constituents of the protein molecule itself Mueller (91) showed that wood charcoal removed from beef heart infusion some component needed for development of the

streptococcus The infusion could be reactivated by addition of small quantities of peptone or acid hydrolysates of casein and edestin The activating material was separated by precipitation with heavy metals into two fractions which exhibited activity only after mixing

Whitehead (165) applied precipitation with phosphotungstic acid to a tryptic digest of casein Substances necessary for growth of a hemolytic streptococcus were removed with the precipitate but were not effective in supporting growth unless small quantities of the filtrate were also added A further separation was accomplished by extraction with butyl alcohol Hosoya and Kuroya (47) reported that an alcoholic extract of rice bran supplied something needed by hemolytic streptococci and that this material accompanied vitamin B McLeod and Wyon (85) attempted to determine the property of fresh blood and serum which promoted growth of pneumococci and meningococci This property of serum could not be extracted by butanol, and digestion of serum with trypsin destroyed it They believed the effect of serum was a phenomenon of the colloidal state Recently Rane and Subbarow (114a) reported that a mixture of glutathione, thiochrome, flavin, nicotinic acid, betaine, glucosamine and a calcium-alcoholic precipitate of highly purified liver extract, in a deficient basal medium, provided almost optimum conditions for growth of the Dochez NY5 strain of hemolytic streptococcus Omission of one or more of these factors decreased the amount of growth

The saprophytic streptococci, particularly those of importance to the dairy industry, have also received some attention Orla-Jensen, Otte, and Snog-Kjaer (108) found that the active material in skim milk could be removed by adsorption on charcoal or fuller's earth and elution with a methanol-pyridine solution This growth-promoting activity appeared to be due to several factors, one of which could be replaced by riboflavin Wood, Andersen and Werkman (175) reported that growth of *Streptococcus paracitrovorous* was improved by the addition of riboflavin

Working with several representative streptococci, Hutner (50) found that at least one factor could be removed from depro-

teinized milk by adsorption with certain brands of fuller's earth The growth-promoting activity thus removed could not be replaced by the addition of pure compounds such as thiamin, riboflavin, uracil or guanine The finding with respect to riboflavin is contrary to that of Orla-Jensen Rahn and Hegarty (114) noted that lactic acid production by centrifuged and washed cells of *Streptococcus lactis* was increased regularly by the addition of 0 002 per cent nicotinic acid Small amounts of ascorbic acid stimulated injured or exhausted cells Adenine, inositol and riboflavin produced no effect In several of these studies lactic acid-producing streptococci have been used along with the lactobacilli A further consideration of this work is presented in a later section dealing with the *Lactobacillus* group

Knowledge of the growth-accessory factors for the streptococci has in general not progressed much beyond the stage of impure tissue extracts Although there have been isolated reports of the effect of chemically pure compounds, their efficacy in promoting growth of the various types of streptococci is not established at present

Staphylococcus

On fractionation of meat extract, Hughes (49) obtained an "activator" for staphylococci This was effective in promoting growth in Uschinsky's medium or in a casein digest medium, in which freshly isolated strains were incapable of multiplication The active material was concentrated to a point where the addition of 0001 milligram to 5 cc of casein digest supported ready development It was heat stable at pH 7 0, soluble in water, alcohol and acetone, but insoluble in ether and benzene It dialyzed through collodion membranes and disappeared on acid hydrolysis of the meat extract An apparently similar material was obtained from yeast extract ("marmite") by Knight (51) Typical strains of *Staphylococcus aureus* grew readily upon the addition of small amounts of this material to a basal medium of hydrolyzed gelatin, amino acids and glucose

Further studies of this fraction by Knight, Fildes and associates have been instrumental in throwing light on the real nature of the active materials and their work constitutes an important

contribution to our knowledge of essential nutritive substances for bacteria A high-vacuum distillate containing the active growth factor was used for further analysis Biological indications from other sources, together with chemical and spectrographic evidence of their own, suggested the testing of cozymase, nicotinic acid, nicotinamide, and thiamin as definite compounds to replace the unknown yeast factor Later, in a study of the absorption spectrum of the high-vacuum distillate secured from yeast, Holiday (45) concluded that nicotinic acid was present in the free state in the yeast concentrate

It was found that the factor was a complex and that one component of it could be replaced by nicotinic acid (or nicotinamide) and the other by thiamin (Knight, 53) These two substances were not effective when added singly, but when supplied together a ready development of *Staphylococcus aureus* was secured On substituting a collection of amino acids for the gelatin hydrolysate previously used, the organism was then grown in a medium the constituents of which were chemical entities of known structure (Fildes et al , 36)

The small amounts of nicotinic acid or its amide and of thiamin which sufficed for development were quite striking (Knight, 53, 54) A concentration of the amide of $6\,6 \times 10^{-7}$ M (0 08 microgram per cubic centimeter of medium) supported maximum growth in 27 hours in the synthetic medium, while light but still detectable growth was secured in the presence of $2\,6 \times 10^{-8}$ M amide The smallest amount of thiamin which supported maximum development was about $1\,0 \times 10^{-8}$ M while $5\,0 \times 10^{-10}$ M produced a detectable effect These amounts are equivalent to 0 003 and 0 00015 microgram per cubic centimeter of medium, respectively

The activity of compounds related to thiamin was also studied by Knight (54, 55a) The components of the thiamin molecule, namely the pyrimidine plus the thiazole, were effective in place of the complete molecule (in the presence of appropriate amounts of nicotinamide) However, a number of other closely related compounds could not be substituted, indicating a high degree of specificity in the requirement of this organism

For anaerobic growth of the staphylococcus, Richardson (121)

reported that uracil was required, a concentration of M/20,000 being most effective Twenty-one other related compounds were studied but showed no comparable effect This striking specificity of uracil, it was suggested, indicates that the compound must be widely distributed in nature and that it exerts an effect which cannot be reproduced by adenine and its derivatives

Another item concerning growth of the staphylococcus has been added by van Wagtendonk (cited by Kogl, 57) The addition of Kogl's "biotin" in the form of its methyl ester resulted in more luxuriant growth Amounts of 0 005 and 0 05 microgram of the biotin ester produced a three- to four-fold stimulation of growth when added along with small amounts of thiamin and nicotinic acid Biotin ester alone gave a slight increase in growth In this instance a compound has been added which has been obtained in crystalline form although the chemical structure is unknown

These results indicate that for best growth of at least some strains of staphylococci, something more than thiamin and nicotinic acid is needed Since Knight reported very good growth of his *Staphylococcus aureus* in approximately 24 hours, it is possible that some strains may not need biotin or are able to synthesize it themselves In a recent confirmation of Knight's work, the writers and associates (63) found that a strain of *Staphylococcus albus* developed in a synthetic medium containing thiamin and nicotinic acid, but growth was considerably slower than that secured in broth or after the addition of fractions of a spleen preparation to the synthetic medium Evidently something else was needed by this strain for optimum growth, though whether this need could be filled by biotin is not known

Whether or not another substance is needed for best growth of the staphylococcus, it has been demonstrated by Knight that this organism, which formerly failed to grow in synthetic media, can now be grown successfully by the use of chemically definite compounds It is of interest to note that of the growth factors required by the staphylococcus, the two for which the chemical structure is definitely known (thiamin and nicotinic acid) are also needed for the normal functioning of the mammalian organism

The diphtheria bacillus

The nutritive requirements of this organism have been the subject of many studies Most strains refuse to grow in synthetic media composed of the usual amino acids, salts and sugar Evidently something else is needed for development Separation and identification of these additional growth factors have been the objectives of an interesting series of reports by Mueller and associates Starting with a suitable medium containing either meat extract or other extracts of fresh tissues, they attempted to separate the components needed for cell multiplication (98, 93) Most of the growth-promoting activity of liver preparations was found in an alcohol filtrate of an aqueous extract, and substances essential for growth could be removed from such a solution by adsorption with wood charcoal and later recovered from the charcoal by elution with acid alcohol (93)

Further studies of the liver eluate by Mueller showed that the active materials could be separated into two fractions by repeated extraction of acid solutions with ether Both fractions were required for the full growth-stimulating effect (99) The ether-extractable substance from liver could be replaced by concentrates of urine (cow and horse) On further investigation of this source and the use of fractional distillation with the Rittenberg apparatus an active substance was obtained and identified as pimelic acid, $C_5H_{10}(COOH)_2$ (94)

When added to a basic medium consisting of casein hydrolysate, cystine, glutamic acid, sodium lactate and inorganic salts together with the ether-insoluble liver fraction, either the isolated product or synthetic pimelic acid produced a two- to three-fold stimulation of growth Quantitative determinations of bacterial nitrogen (92) showed that the stimulating effect of pimelic acid became evident at a concentration of about 0 005 microgram per cubic centimeter of medium and reached a maximum in the presence of five to ten times this amount (94) Other dibasic acids of the same series, from oxalic up to azelaic, exerted no growth-stimulating effect

Following the identification of pimelic acid, attention was next turned to the ether-insoluble fraction of liver extract This

material, after combined esterification and acetylation, was subjected to fractional distillation, and growth-promoting activity appeared in both the lowest-boiling and the highest-boiling fractions Nicotinic acid was substituted for the low-boiling fraction and showed the same growth-promoting activity The most striking effect of nicotinic acid was exerted in a concentration of about 1 0 microgram per cubic centimeter of medium while approximately ten times as much nicotinamide was required to produce a comparable effect (95)

The high-boiling fraction of the vacuum distillate remained as the only source of unidentified material and this was next subjected to examination by Mueller and Cohen (97) Chemical evidence indicated the presence of amino acids and it was found that β-alanine, which had been shown by Williams and Rohrman (171) to exert a growth-promoting effect on yeast, could be substituted for the high-boiling material β-Alanine produced its maximal effect in a concentration of about 1 microgram per cubic centimeter of medium *l*-Carnosine (β-alanyl histidine) was also effective but a greater concentration was required (96)

As a result of these studies it was shown that three substances of known chemical structure could be substituted for the hitherto unknown materials in extracts of liver and other tissues In comparing the effect of these three compounds on the growth of four strains of diphtheria bacilli it was evident that each of them, when supplied alone in the basal casein-hydrolysate medium, exerted no appreciable growth-promoting effect β-Alanine and nicotinic acid together were quite effective, and for some strains pimelic acid exerted an added stimulative effect (97) On substituting amino acid mixtures for the basal medium of hydrolyzed casein, several cultures of the Park 8 strain developed readily and produced a potent toxin in a medium of definite chemical composition (110) These reports by Mueller and associates provide an excellent example of the value of intensive and thorough search for the substances in tissue infusions which are required by some bacteria, and they constitute an important contribution to our knowledge of the nutritive requirements of bacteria

The rôle of β-alanine and nicotinic acid was recently confirmed by other workers (63) though β-alanine appeared to be the more important of the two substances insofar as one Park 8 culture was concerned Addition of β-alanine alone to a basal medium of amino acids, dextrose and mineral salts resulted in growth of a strain of the organism which failed to develop without the β-alanine In contrast, nicotinic acid or pimelic acid alone did not support growth but the former stimulated development when added with β-alanine

While Mueller's work has done much to clarify our knowledge of the usual requirements of this organism, it should be added that apparently some strains of diphtheria bacilli either do not require the foregoing compounds or else are able to synthesize them, for evidence has appeared from time to time that occasional strains of the organism may be cultivated in ordinary amino acid synthetic media and in this earlier work β-alanine and nicotinic acid were of course not used Typical of such reports are those of Braun, Hofmeier and Mundel (7), of Maver (84) and of Wadsworth and Wheeler (162) The last mentioned investigators obtained growth of thirteen out of twenty recently isolated virulent strains in a synthetic medium which did not contain β-alanine and nicotinic acid Development of the cultures was slow, but could be carried through successive transplants and weak toxin was produced

Dysentery bacilli

Many strains of dysentery bacilli fail to develop in the usual synthetic media composed of amino acids, glucose and inorganic salts, evidently other substances or conditions are required Upon addition of small amounts of tissue extracts to such a medium, the cultures usually develop readily The growth-promoting substances in veal infusion, yeast, and other animal and plant tissues can be obtained in impure form by charcoal adsorption (65) They can also be partially purified by treatment of tissue infusions with solutions of heavy metals which precipitate inert material, but do not precipitate the growth factors (127)

Recently it has been shown by Koser, Dorfman and Saunders

(61) that nicotinic acid or nicotinamide can be substituted for the fractions from tissue extracts and thus it is now possible to secure growth in a medium of definite chemical composition Amounts of 0 1 microgram of nicotinic acid per cubic centimeter of synthetic medium caused prompt growth with pronounced turbidity of a number of Flexner and Sonne strains, while 0 01, 0 004 or at times even 0 002 microgram per cubic centimeter sufficed for slower and scantier development Whether the growth-promoting property of the tissue extracts is due to nicotinic acid or to the amide is not known, since the presence of these compounds has not yet been definitely established in these preparations

It is of interest that for the dysentery bacilli nicotinic acid, or its amide, seems to be the only substance needed in addition to amino acids, glucose and salts In the case of the staphylococci, it will be recalled, both nicotinic acid (or the amide) and thiamin were needed, and for the diphtheria bacillus a combination of nicotinic acid and β-alanine gave the best results

Brucella

Growth-promoting substances in extracts of yeast and beef liver were precipitated with phosphotungstic acid and found to be remarkably stable on heating in the presence of acid or alkali (48) Koser and Saunders (65) found that growth-promoting activity for *Brucella* could be removed from various plant and animal tissue extracts by charcoal adsorption and recovered by subsequent elution with alcohol or acetone The active substances could also be concentrated by precipitation of inert material with heavy metals Substitution of various definite compounds for the active fractions of tissue extracts has not been successful Thus, the addition of nicotinic acid, thiamin, riboflavin, β-alanine and other compounds to a synthetic medium was not followed by growth of a *Brucella abortus* culture (63)

Development of some *Brucella* cultures in synthetic media without the addition of added growth factors has been reported by ZoBell and Meyer (179), though even the most promising of their synthetic media were far from satisfactory Growth was slow and some cultures refused to multiply in the second trans-

fer Furthermore, from one hundred thousand to a million cells per cubic centimeter of synthetic medium were needed to insure positive results The inability of small numbers of cells to initiate growth suggests that other factors or conditions were needed

The hemophilic bacteria

Studies of the accessory requirements of *Hemophilus influenzae* and allied hemophilic types have received more attention and are better known to most bacteriologists than those dealing with other groups of microorganisms The reports of Davis (20, 21), Thjotta and Avery (153, 154, 155), Fildes (32) and others demonstrated that two substances were necessary for development of Pfeiffer's bacillus One of these was associated with the hemoglobin of blood and the other occurred in a variety of plant and animal tissues or in the extracts of microorganisms Extracts of potato apparently contained both substances From these studies there emerged the now-familiar V and X factors Separately these factors are not sufficient for cell proliferation of *H influenzae* but when supplied together in ordinary culture media prompt development occurs

The V factor is thermo-labile, diffuses through parchment membranes and is easily destroyed in alkaline solution Its potency is lowered or it may be completely inactivated on contact with fresh serum It is produced by a number of bacteria as well as by yeasts and molds and is found in many plant and animal tissues The X factor is relatively thermo-stable and is associated with the non-containing fraction of hemoglobin It may be replaced by hematin It occurs in plant tissue, especially potato, and is probably elaborated by some bacteria It is often, though not always, associated with peroxidase activity and it has been suggested by several workers that its action is of a catalytic nature, accelerating the transfer of oxygen from peroxides in the medium or from the atmosphere to the bacillus The essential points with respect to the V and X factors were confirmed and extended by a number of workers during the several years following 1921 No detailed account of these

results need be given here since this material has been covered in previous reviews (141, 111, 52)

The requirements of other organisms of the influenza group have been studied to some extent Certain representatives of this group required only the V factor and were termed *H parainfluenzae* by Rivers (123) Among these influenza-like bacilli there were encountered both hemolytic and non-hemolytic strains which possessed the common characteristic of being able to develop in the presence of the V but without the X factor (33, 160) In contrast to these organisms are the so-called *B hemoglobinophilus canis* of Friedberger which requires only the X factor (Rivers, 122) and Ducrey's bacillus of soft chancre (*Hemophilus ducreyi*), which according to Lwoff and Pirosky (79) needs for its growth the X factor (hemin) but not the V factor

More recently some additional information on the nature of the V factor has appeared The earlier suggestions that the V factor might be vitamin C (ascorbic acid) appear to have been definitely ruled out by Meyer (86) Studies on the coenzyme of Warburg and the cozymase of Harden and Young have afforded the basis for an important step in our understanding of the V factor Lwoff and Lwoff (76) found that either the coenzyme or the cozymase could be substituted for the unknown V factor and *H parainfluenzae* then developed readily in peptone solution It is interesting that nicotinic acid, its amide, diethylamide and adenylic acid could not be substituted for the coenzyme or V factor This represents a contrast to the staphylococci and the dysentery bacilli which either are able to synthesize the codehydrogenase or can utilize the constituent parts as such

It was found also (77) that the codehydrogenases had no influence upon the speed of reduction of methylene blue or of oxygen uptake by cells of *H parainfluenzae* grown in the presence of an excess of V factor, but did increase these processes by cells grown in the presence of small amounts of V factor When approaching the limit of active dilutions, the action of the codehydrogenases was quantitative Evidently the physiological function of V factor is that of a catalyst in cell oxidations Similar evidence was submitted by Lwoff and Lwoff (78) with respect to

the rôle of hemin (X factor) They believe that the function of hemin as a growth factor is in the formation of respiratory enzyme systems such as cytochrome, cytochromoxidase, catalase and peroxidase These interesting contributions supply evidence concerning not only the chemical nature of the hitherto mysterious V factor, but also the rôle of both the V and X factors

Acid-fast bacteria

Johne's bacillus usually fails to develop even in the more complex media, unless killed cells or extracts of other acid-fast bacteria are added Twort and Ingram (157) attempted to isolate the substance essential for Johne's bacillus from cells of other acid-fast types, such as *Mycobacterium phlei* Several extracts were prepared and from one of them a small amount of the active substance was precipitated with barium salts Further purification was not attained The growth-promoting activity was not destroyed by autoclaving While it is uncertain whether the so-called "essential substance" studied by Twort and Ingram over twenty-five years ago can be classed with the accessory factors, the work nevertheless possesses considerable interest as being one of the earliest attempts to isolate growth substances from complex mixtures

Tubercle bacillus Of the various pathogenic bacteria the tubercle bacillus is often regarded as one of the less exacting in its nutritive requirements, since many strains may be grown in the simpler synthetic media, of which Long's is probably the best known There is evidence, however, that additional substances are required for maximum development and that the tubercle bacillus is unable to initiate growth in Long's medium unless the solution is seeded with large numbers of cells Uyei (159) states that growth in Long's medium occurred only when the inoculum contains 1 milligram of cells (about five billion), whereas in Petroff's glycerol-egg medium and in a potato-glycerol medium development of cultures could be secured with inocula of 0 001 milligram and 0 000,000,001 milligram, respectively

Addition of a yeast preparation or of orange, tomato or cabbage juice to Long's medium increased markedly the amount of growth

of both human and bovine types of the bacilli after 2 and 3 weeks of incubation (Uyei, 158) Of the several interpretations which may be made from such an observation, Uyei emphasized the supposed vitamin-like nature of the accelerating substances and suggested a relationship to vitamin B (complex) Uyei (159) also studied the nature of the growth-promoting principles of the potato and reported that the active substances could not be extracted with acetone, alcohol, or ether

Not only is the nature of growth accessories for the acid-fast organisms unknown, but there is doubt as to whether such substances are required for the better-known representatives of the group

The anaerobic spore-formers genus Clostridium

A "vitamin" necessary for cell proliferation of *Clostridium sporogenes* was described by Knight and Fildes (55) Cultures of the anaerobe failed to develop in an acid hydrolysate of photographic gelatin supplemented by tryptophane, sodium citrate, thioglycollic acid and inorganic salts unless small amounts of the "sporogenes vitamin" were also added Two-tenths of a microgram in 10 cc of basal medium was sufficient for growth just visible to the eye The active material was obtained in the form of a yellow gum from yeast and could also be obtained from human urine The same substance, or something capable of replacing it, was also synthesized by certain microorganisms, notably *Salmonella aertrycke*, the tubercle bacillus, and *Aspergillus versicolor*

Substitution of an amino acid mixture for the gelatin hydrolysate was made possible through the study of Fildes and Richardson (35) and development of *Cl sporogenes* was then secured in a medium the only unknown component of which was the "vitamin" Fildes (34) has presented evidence to show that the "sporogenes vitamin" is also needed by many strains of *Cl botulinum* Pappenheimer (109) made a further study of the chemical properties of this growth factor Highly active preparations were secured but the substance could not be obtained in crystalline form The material had the properties of an unsaturated hy-

droxy-acid of molecular weight about 200, and the formula $C_{11}H_{14}O_4$ or $C_{11}H_{11}O_4$ was suggested The factor was considered to be distinct from the plant auxins, Williams' pantothenic acid, the staphylococcus factor of Hughes, and the bios of Kogl

Propionic acid bacteria lactobacilli, butyl alcohol bacteria *Propionic acid bacteria* These microorganisms are usually considered to be fastidious in their growth requirements since they do not multiply readily in the ordinary peptone medium, but develop much more rapidly upon the addition of milk whey, yeast extracts or tissue extracts Van Niel (161) believed the superior fermentation obtained in the presence of yeast extracts and yeast autolysates could not be ascribed to differences in nitrogen content or buffer capacity and suggested that accessory substances in yeast might play an important part The stimulative effect of potato extract, orange juice and yeast-water on glucose fermentation and acid production by these organisms was studied especially by Fromageot and Tatum (38) and by Tatum, Peterson and Fred (150) Evidence indicated that the stimulative activity of potato extract was not due primarily to available nitrogen content or buffering capacity By use of the Neuberg reagent (mercuric acetate and sodium carbonate) the potato extract was separated into two fractions both of which were needed for maximum stimulation The Neuberg filtrate fraction was believed to contain some accessory substance other than mineral salts, since after ignition the ash did not produce the complete stimulative effect (150) The effect of the Neuberg precipitate was due primarily to ammonium nitrogen and asparagine Ammonium nitrogen was utilized in the presence of the proper growth factors (151)

In a continuation of this work, yeast extract was used as the source of growth stimulant and from it Wood, Tatum and Peterson (176) obtained a fraction apparently essential for growth of various strains of propionic acid bacteria in a glucose-ammonium sulphate medium This factor was acidic in nature, non-volatile and could be extracted with ether It could not be replaced by other biologically active substances, namely thiamin,

the flavin fraction from liver, the sporogenes vitamin of Knight, Williams' pantothenic acid, indoleacetic acid, inositol, or nicotinamide It should be emphasized, however, that the propionic cultures did not grow indefinitely in the glucose-ammonium sulphate-yeast factor medium, indicating the need for some additional material This was supplied by hydrolyzed casein or by unhydrolyzed casein, egg albumin or milk powder From these sources, as well as from yeast extract, the active material could be extracted with alcohol and acetone It was neither an amino acid nor a part of a protein molecule (Tatum, Wood and Peterson, 152) The solubilities and stability of the active fraction resembled those of thiamin and this similarity suggested substitution of the pure vitamin Two different samples of thiamin were found to be capable of completely replacing the extract One sample was effective in amounts of 0 005 microgram per cubic centimeter of medium while 0 05 microgram of the other lot was required Inositol, pantothenic acid, ascorbic acid, hepatoflavin, nicotinamide and indoleacetic acid were not effective in replacing the extracted material Here we have an interesting instance of the replacement of an unknown growth-stimulating material by a known substance of definite chemical composition, thus advancing materially our knowledge of the physiological requirements of the propionic acid bacteria Evidently, at least one other substance is needed and it is contained in the acid-ether extract of yeast or potato

There is also evidence that riboflavin is a stimulant for propionic acid bacteria On fractionating yeast extract, Lava, Ross and Blanchard (69) found the B_2-containing portion to be the most active in stimulating acid production This was confirmed with pure riboflavin by Wood, Andersen and Werkman (175) They also found (175a) that the factor in the ether extract of yeast extract was essential for all cultures of propionic acid bacteria This factor could not be replaced by a mixture of nicotinic acid, thiamin, pimelic acid, uracil, β-alanine and "pantothenic acid" Riboflavin and thiamin stimulated growth but were not essential

Lactobacilli Orla-Jensen, Otte, and Snog-Kjaer (108) stated

that riboflavin and one or more other "activators" are necessary for normal development of certain lactic acid bacteria Their conclusion that one of these substances is pantothenic acid seems questionable, however Their finding concerning riboflavin was confirmed (175) Other unknown substances were also needed and the requirements varied somewhat with different lactic acid types Unknown factors in the basal medium (175) were the ether-soluble component from yeast and hydrolyzed casein Seventeen purified amino acids did not satisfactorily replace the hydrolyzed casein

Snell, Tatum and Peterson (145) reported that two unknown factors appeared to be necessary for attainment of luxuriant growth by *Lactobacillus delbrucki* in a hydrolyzed casein medium containing added tryptophane and a fermentable carbohydrate One of these factors occurred in the Neuberg filtrate fraction or in an acid-ether extract of crude potato extract The evidence suggested an acid of fairly low molecular weight The second factor occurred in peptone, was basic and could be precipitated with Neuberg's reagent and with lead acetate and ammonia Liver extract contained both of the growth stimulants, or other substances capable of replacing them

In a later report Snell, Strong and Peterson (144) found one of the factors in liver to be an acidic, ether-extractable organic substance The maximum effect of this fraction was attained in the presence of 0 1 to 0 3 microgram per cubic centimeter of basal medium, though its effect was detectable with amounts as small as 0 003 microgram The basal medium contained riboflavin, which also exerted a stimulating effect in small amounts, and sodium acetate in addition to other more commonly used substances A number of known compounds were tested but failed to replace the fraction from liver These were auxin-*a*, 3-indoleacetic acid, pimelic acid, pyruvic acid, uracil, and combinations of nicotinamide and thiamin The relationship of this substance from liver to those previously described and to the ether-extractable substance for propionic acid bacteria is not clear at the present time

Butyl alcohol bacteria Recently Brown, Wood and Werkman

(9) obtained an acidic, ether-soluble fraction from yeast extract which was essential for vigorous growth of butyl alcohol organisms in a medium consisting of hydrolyzed casein, tryptophane, ammonium sulphate, glucose and inorganic salts When a mixture of 18 purified amino acids was substituted for the hydrolyzed casein the organisms refused to grow The hydrolyzed casein apparently contained a second unknown factor or else an essential amino acid in addition to those used Here again, as in much of the previous work reviewed in this section, it appears that at least two substances are needed, one occurring in the acid-ether extract of yeast and the other in hydrolyzed casein Werkman and associates (9) state that the latter is not thiamin

Insofar as the requirements have been elucidated, it is evident that these fermentative bacteria, which are not associated with invasion of animal tissues, nevertheless require some of the vitamins which are essential for the higher animal

Nitrogen-fixing bacteria

Growth of the various types of *Rhizobium* in synthetic media is usually negligible if pure, ordinary ingredients are used Upon the addition of small amounts of yeast extract the cultures develop readily and evidence has been advanced, notably by Allison and Hoover (1, 46), that the effect of the yeast can be attributed to the presence of small amounts of a growth factor This substance they termed "coenzyme R" It was found to be present especially in yeast, cane molasses, natural humic acid, commercial egg albumin, and commercial sucrose It could be obtained along with impurities by extraction of commercial sucrose or dried cane molasses with absolute alcohol Small amounts of such extracts, when added to the usual synthetic medium, led to good development of cultures of the root-nodule organisms (1) The extracts also stimulated the rate of respiration as determined in the Warburg apparatus

Hoover and Allison (46) obtained apparently the same factor in more concentrated form from *Azotobacter* cultures which had evidently synthesized it Attempts to obtain it in crystalline form were not successful The substance was dialyzable and

quite heat-stable It was not identical with Williams' pantothenic acid Cystine and related reducing substances, inositol, synthetic iron humates, and various nucleotides could not be substituted for it They also report (2) that the growth response of the nodule bacteria to natural humic acid is due almost wholly to this factor and not to the available iron content The presence of a somewhat similar substance in brown sugar and in calcium sucrate has been noted by Clark (17) This substance was responsible for growth acceleration of *Rhizobium trifolii* It was destroyed by ashing, was dialyzable, was adsorbed by charcoal and was reported to resemble in some respects the bios complex of yeast

It is difficult to correlate these reports and at present no conclusion can be reached concerning the nature of any growth factor which might be needed by *Rhizobium* or other nitrogen-fixing soil organisms

Streptothrix

Attempts were made by Reader and associates to separate growth-promoting substances for *Streptothrix corallinus* from an enzymic digest of beef No pure compound was obtained but the active material was stated (116) to be organic, water-soluble, ether-insoluble, dialyzable, stable to alkali in the purest preparations, and not precipitated by neutral or basic lead acetate It was not identical with vitamin B_1 or B_2 preparations (112), but a similarity in constitution to B_1 was suggested In later work (117) it was found that mannitol, but not the other alcohols commonly used in bacteriological work, considerably increased the mass of growth when added to the salt-sugar medium together with a growth factor preparation It was believed that the mannitol acted as a specific source of food rather than as an additive growth-promoting factor, and a similar interpretation was suggested for the effect of *i*-inositol (bios I) upon yeast

The relation of this streptothrix growth substance to the other bacterial growth-promoting factors is not clear Repetition of the tests with pure preparations of thiamin and the other compounds now available would be of interest

YEASTS

A consideration of growth accessory substances for the yeasts revolves largely about the question of "bios," the term first applied in 1901 by Wildiers (166) to designate material in yeast extract which was needed for normal cell proliferation of *Saccharomyces cerevisiae* in a synthetic medium Wildiers and a little later Devloo (22) recorded some of the physical and chemical properties of bios and stated that it was not present in yeast ash The study of the accessory growth substances for microorganisms may be said to have started with this work

It will be recalled that up to this time Pasteur's opinion had prevailed, namely that yeast could be cultivated readily in a solution consisting only of sugar, an ammonium salt and ash of yeast To this Liebig had objected and offered evidence to the contrary, though the weight of opinion continued to favor Pasteur's view Wildiers proposed a possible explanation for these differences based on size of the inoculum and consequent carrying over of bios He evidently considered his term bios to be only a tentative one, expressing the hope that a chemical name might later replace it In this he appears to have been years ahead of his time for a quarter of a century was to elapse before any real progress was made in this direction

Wildiers' ideas were soon challenged and for a few years a controversy ensued concerning his interpretations and methods This has been reviewed by Tanner (149) and Buchanan and Fulmer (10) The discussion of bios largely disappeared for a time, only to be revived some years later when it was maintained that vitamin B and bios were the same and that the stimulation of yeast growth by bios supplied a quantitative method of assaying vitamin B (168) It was soon shown that this assumption in its original form was incorrect However, a renewed impetus was given and much of our present knowledge of bios is due to the intensive studies started at this time by several groups of investigators, especially those associated with Fulmer, Miller, and Williams

Evidence shortly appeared to show that bios was in reality a complex of a number of substances and that the combination of

all of them was often necessary to exert the stimulating effect (72, 39, 173) Similar situations have been encountered more recently in the study of other growth factors Along with recognition of the multiple nature of bios further complications arose when it was realized that different yeasts possessed quite different nutritive requirements In the earlier work there had been a tendency to regard yeast as a single entity Some of the contradictory statements found in the literature undoubtedly were due to this failure to recognize the marked differences between species and strains This situation was remedied, however, as attention was directed to the differing nutritive requirements of various yeasts Lucas (72) found that different strains of yeast varied in their response to bios, and similar findings were reported by Williams, Wilson and von der Abe (173), Copping (18) and others In the more recent studies this difference in requirements has been well recognized

Aside from the yeast cell itself, many other sources of bios-like growth stimulants have been reported Wildiers (166) originally called attention to several of these sources, and more recently the presence of growth factors for yeast has been noted in alfalfa (39), the buds and leaves of a number of plants (19), oat coleoptiles (27), tomato juice (28, 88) and commercial sugars (43) All the evidence indicates a widespread distribution in nature

Regarding the chemistry of the bios complex, the separation accomplished by Lucas (72) seems to have been the basis for much of the subsequent work He obtained two fractions, bios I and bios II, by treating with alcoholic barium hydroxide Separately each fraction possessed little activity but when combined the original activity was restored In subsequent work by Eastcott (25) the active principle of bios I was identified as *i*-inositol

Later work showed that bios II was not a single entity and several groups of investigators fractionated it by one method or another Miller, Eastcott and Sparling (89) recognized a bios II A and bios II B Crude bios II B contains a new constituent provisionally named bios VII (88) It was reported (87)

that II A could be replaced by β-alanine and leucine Also a bios V which appeared to be necessary for a certain strain of yeast was reported by Farrel (28) The bios V, however, did not increase the crop of *Saccharomyces cerevisiae* or several other common yeasts Bios V can apparently be replaced by thiamin (88)

In the meantime Williams and Roehm (170) found that thiamin stimulated growth of some yeasts and pointed out that it possessed certain properties in common with one of the components of bios II In addition, there was evidence of marked growth-promoting activity in another fraction In further work Williams and associates (169) reported the presence and partial purification of an acidic substance which markedly stimulated growth This substance was of widespread occurrence in nature and was called "pantothenic acid" Small amounts of pantothenic acid alone, when added to a basal medium, exerted some growth-promoting effect, but the activity was enhanced by addition of i-inositol or thiamin or both (Williams and Saunders, 172) Richards (120) has reported that pantothenic acid stimulates yeast growth by shortening the generation time This was seen particularly when the seed yeast came from older cultures There was less effect on the crop

Williams and Rohrman (171) added β-alanine to the list of growth-promoting compounds When incorporated in a basal medium containing salts, sugar and inositol, 0 08 microgram per cubic centimeter of β-alanine produced growth stimulation of five yeast strains The addition of aspartic acid to the medium resulted in a still larger yeast crop, while one of the five strains also required thiamin

Recently Kogl and Tonnis (59) announced the isolation of a substance called "biotin" which was obtained in crystalline form as its methyl ester This was isolated from what constituted part of the bios II complex (the fraction adsorbed on charcoal) Biotin possesses a marked stimulating effect on yeast growth, a dilution of one part in 4×10^{11} producing a perceptible effect, while one part in 4×10^{10} caused a more distinct stimulation

In much of the foregoing work various strains of *Saccharomyces*

were used Schopfer (134, 135) has recently studied the requirements of several of the torulae, *Rhodotorula rubra* and *R flava* These yeasts required thiamin for satisfactory development, and maximum growth was obtained with about 0 4 microgram in 25 cc of synthetic medium The pyrimidine component of thiamin could substitute for the whole molecule, the thiazole component was practically without effect Inositol and pantothenic acid or combinations of both produced no growth-promoting effect upon these two species Further differences in the requirements of different yeast strains with respect to thiamin and its two component ring structures have been reported by Schultz, Atkin and Frey (140)

In an interesting report Sperti, Loofbourow and Dwyer (146) have directed attention to the liberation by injured cells of substances affecting growth Cells of *Saccharomyces cerevisiae* injured by ultraviolet irradiation produced substances which stimulated cell proliferation Apparently the effect was due not merely to substances found in normal cells but to products elaborated by the injured, living, cells as a definite response to injury These results are of interest in relation to similar evidence concerning the proliferation of cells in tissue cultures Also they may have a general bearing upon the preservation of communities of microorganisms following injury to some of the cells of the community Norris and Kreke (105) have presented evidence to show that the factors affecting growth, fermentation and respiration of *Saccharomyces cerevisiae* are not one and the same substance Using malt combings as a source of bios, they showed that factors which affect these different cell activities could be concentrated in different fractions

To sum up the evidence, it is quite apparent that the yeasts as a group vary as widely in their requirements of accessory growth substances as do the bacteria Some yeasts can develop through continuous transplants in simple synthetic media and evidently are able to synthesize all needed compounds Others are stimulated in greater or less degree by additions of *i*-inositol, thiamin, β-alanine, "biotin" and "pantothenic acid," depending upon their inability to obtain by synthetic or other processes one or more of

these materials Doubtless still other compounds will be found to be needed by some of the more exacting species

MOLDS AND HIGHER FUNGI (EUMYCETES)

Although many of the fungi develop readily in very simple solutions containing inorganic nitrogen, a sugar and mineral salts, it is well known that others are more exacting in their requirements and complex mixtures such as peptone, protein hydrolysates and tissue extracts must be supplied for their successful cultivation Some of them, indeed, are worthy rivals of the more exacting bacteria in their nutritive requirements As in the case of the bacteria, a number of suggestions were advanced from time to time that vitamin-like substances were needed by these forms (Linossier 71, Willaman 167, Lepeschkin 70) At the time, fifteen to twenty years ago, the evidence for this was necessarily vague More recently, with increasing knowledge of the vitamins and particularly of the vitamin B complex, this suggestion has been subjected to more direct experimental proof

Nematospora gossypii was one of the first to be investigated systematically Farries and Bell (30) reported that it required an "accessory factor" which could be obtained in impure form from egg white, crude casein and other sources Its exact chemical nature was not determined This work was confirmed by Buston and Pramanik (16), who separated the factor into two fractions by precipitation with barium hydroxide and alcohol Neither fraction was active in the absence of the other The active component of one fraction was identified as i-inositol, while the other was concentrated to a considerable degree but not identified (15) In the presence of inositol and a "second accessory factor" from lentils *N gossypii* grew on a medium whose sole nitrogenous constituent was asparagine or ammonium aspartate (15a) It seems likely that this second fraction contained the biotin of Kogl or a mixture of biotin and thiamin, for Kogl and Fries (58) secured growth of *N gossypii* in a synthetic medium containing these substances together with i-inositol

Recent studies, particularly those of Schopfer, have emphasized the importance of thiamin for a number of the fungi Growth

and zygospore formation of *Phycomyces blakesleeanus* (a *Mucor*) took place in a synthetic medium following the addition of crystalline thiamin In fulfilling the nutrient requirements of this mold, thiamin satisfactorily replaced concentrates prepared from yeast or other sources (129, 12) Riboflavin showed no such effect (12) This work was soon extended to include additional species of molds (130, 131) belonging to the genera *Absidia*, *Parasitella*, *Mucor*, *Pilaria* and others, many of which were found also to require thiamin In contrast to these results *Rhizopus* was inhibited Results obtained with natural thiamin were shortly confirmed by the use of a synthetic preparation, and maximum growth of *Phycomyces* was secured upon the addition of 0 5 microgram to 25 cc of medium (132)

In the investigation of other sources of growth-promoting substances, Schopfer (131) reported that wheat-germ extract apparently contained at least one other factor in addition to thiamin, for small amounts of the extract produced rapid development of *Rhizopus* He also found (133) that extracts of the leaves of many species of higher plants supplied substances producing a similar effect on *Phycomyces* This material could be extracted with alcohol, was thermostable in acid solution and was adsorbed by fuller's earth and animal charcoal In a further study of wheat-germ, Schopfer and Moser (139) described procedures for separation and concentration of several factors showing activity for both *Phycomyces* and *Rhizopus*, especially two factors which they designated "MR" and "MP" Thermostability, resistance to alkali and adsorption by animal charcoal were useful for differentiation It was suggested that MP might be a disintegration product of thiamin Certain other preparations or definite compounds could not replace the wheat-germ factors Thus, pantothenic acid, either alone or with *i*-inositol, was without effect on *Phycomyces* and furthermore it did not augment the action of thiamin Also heteroauxin (3-indoleacetic acid) had no effect on *Phycomyces* or *Rhizopus* Nielsen and Hartelius (103) reported that *Rhizopus* cultures produce a substance, "Wuchsstoff B," stimulating the growth of *Aspergillus* A stimulant in beer wort for *A niger* and for yeasts

was later subdivided into several components (104) Certain of these stimulants were required for yeasts and were easily oxidized while others affected mold development and were more resistant to oxidation by hydrogen peroxide and potassium permanganate The presence of metals (co-growth substances) was also emphasized by Nielsen (102) The influence of growth factors upon development and nitrogen assimilation of *A niger* was studied by Bunning (11) Thiamin as a rule exerted little growth-promoting effect, while riboflavin led to an increase of about 30 to 40 per cent in the dry weight of the mycelium, though amounts of about 4 to 20 micrograms per cubic centimeter were necessary to bring about this effect Both of the vitamins as well as several unknown growth factors promoted absorption of nitrates by the mold and this in turn was connected with an intensified respiration

Mosher, Williams and associates (90) studied the nutritional requirements of *Trichophyton interdigitale* In addition to rather specialized requirements with respect to inorganic ions and amino acids, this fungus apparently requires at least four accessory substances for satisfactory development These are thiamin, riboflavin, *i*-inositol and Williams' pantothenic acid

A study of the requirements of a number of different fungi, including representatives of the Phycomycetes, Ascomycetes and Basidiomycetes was made by Kogl and Fries (58) Biotin, thiamin and *i*-inositol were tested in a basal medium of glucose, tartrate, and inorganic salts The thiamin requirement of *Phycomyces* was confirmed In addition a number of species of the Ascomycetes and Basidiomycetes were found either to require thiamin or to be stimulated by it A few exceptions to this requirement were also encountered, thus *Nematospora gossypii* required biotin and *i*-inositol for appreciable growth which was further increased by addition of thiamin, while *Lophodermium pinastri* needed biotin and thiamin β-Alanine was without effect on these types Kogl and Fries believed that in those cases where a particular factor was found to be unnecessary it was synthesized by the mold Pairs of fungi with complementary requirements could be grown on a medium without any of the

three factors, although growth was slower under these conditions

Several recent reports have dealt with the effect on molds of the components of the thiamin molecule The need for the pyrimidine and thiazole components appears to differ with various molds In the study of *Phycomyces*, Schopfer and Jung (138) reported that each of these components alone had little or no effect but in combination the effect was identical with that of the whole thiamin molecule Similar results were reported by Sinclair (143) who found also that thiamin diphosphate (cocarboxylase) was about as active as the vitamin itself Robbins and Kavanagh (124) found that a mixture of the thiazole component with a 5-bromomethyl derivative of the usual pyrimidine was as effective as molar equivalent amounts of thiamin The vitamin was therefore believed to be synthesized by the mold from the separate components, since they were required in molecularly equivalent quantities Additional data on the effectiveness of derivatives of thiamin and its two components with respect to microorganisms in general will be discussed in a later section Thus the impure fractions from plant and animal tissues can be replaced in a few instances by small amounts of definite chemical substances Of the compounds thus far demonstrated to possess growth-promoting activity for the fungi, thiamin assumes an important part, as it does with the bacteria and higher forms of life Presumably many of the parasitic fungi are unable to synthesize this molecule or one of its components and so fail to develop Also, there is evidence of the need for a number of interacting factors, the absence of any one of which may lead to complete failure or a marked retardation in development Inositol seems to be needed in some instances, likewise the preparations known as biotin and pantothenic acid Future work will doubtless supply further evidence concerning the nature of these substances and bring to light still others now unrecognized

MISCELLANEOUS STUDIES

In addition to the work on groups of bacteria treated in the preceding sections, several other studies may be mentioned here

From peptone and from blood, Sahyun, Beard and associates (125) obtained in partially purified form "activators" which stimulated cell multiplication of *Escherichia coli* This effect was in addition to that exerted by known amino acids, and the activating substance was not destroyed by growth of the organisms in media containing it Dunn and Salle (24) extracted stimulating agents from rice bran with 60 per cent methanol and 25 per cent ethanol Evidently the rice bran extract also contained food material and inorganic salts The growth of carbohydrate-fermenting organisms was greatly enhanced and it was suggested that the stimulating agent might be carbohydrate in nature, but was not glucose

Koser, Chinn and Saunders (60) found that certain gelatins contain growth factors for many of the commoner pathogens, including such types as hemolytic streptococci from scarlet fever, pneumococci, *Brucella* and others In a synthetic medium, in which these organisms were unable to develop, the addition of some gelatins promoted ready growth of these types A more highly purified photographic gelatin did not support growth under the same conditions

Protozoa While no attempt has been made to review exhaustively the literature dealing with the protozoa, several instances may be cited to show the importance of the accessory growth factors for development of certain of these forms M Lwoff and A Lwoff (80, 73) found that hematin, protohemin, and protoporphyrin could replace an essential substance supplied by blood for cultivation of several trypanosomes of the genera *Strigomonas* and *Leptomonas* Since protoporphyrin contains no iron the trypanosomes can evidently combine this molecule with traces of iron present in the medium and thus construct the iron-containing hematin Lwoff and Dusi (74) and Lwoff and Lwoff (82, 75) have shown that a number of different forms (*Polytomella caeca, Polytoma caudatum, P ocellatum, Chilomonas paramecium, Glaucoma piriformis* and *Strigomonas oncopelti*) need thiamin In addition one or more other factors are probably required by some of these types According to a recent report (83) *Schizotrypanum cruzi* requires ascorbic acid and hematin

THE ROLE OF INORGANIC SALTS IN PROVIDING GROWTH-PROMOTING EFFECTS

One explanation of the growth-promoting properties of tissue extracts is based on the assumption that the effect may be due to the presence of certain inorganic salts, which are needed by the microorganism. Of the enormous literature dealing with the effects of inorganic salts upon microorganisms, the following may be cited as bearing more particularly upon our subject. Webster and Baudisch (163) and Baudisch (4) stressed the importance of certain "active" forms of iron salts and iron oxides which might function as the X factor in the growth of hemophilic bacteria. Reed and Rice (118) secured heavier growth of the tubercle bacillus and of several related acid-fast types in a synthetic medium when small amounts of iron and citrate were added. The citrate prevented precipitation of the iron. Elvehjem (26) emphasized the importance of iron and copper in the growth and metabolism of yeast and suggested that a considerable part of the beneficial action of bios depended upon changes which made iron more available for assimilation. Greaves, ZoBell and Greaves (42) reported that growth of yeasts in a mineral salt-sugar solution was increased by minute amounts of iodine. Richards (119) stressed the importance of thallium and expressed the belief that this element may be one of the growth stimulants for yeast that have been referred to as bios. Thallium in varying amounts was present as an impurity in different brands of asparagine. Burk, Lineweaver and Horner (13) reported that growth stimulation of *Azotobacter* by humic acid was due to the iron content of the latter and that natural humic acid could be replaced by several organic or inorganic iron compounds. Thorne and Walker (156) found that growth of several species of *Rhizobium* in a purified sucrose-nitrate medium was greatly increased by the addition of small amounts of iron, especially ferric chloride. The importance of molybdenum and zinc for development of *Aspergillus niger* was emphasized by Steinberg (147). The decreased yield of mold growth obtained when purified sucrose was used in a synthetic medium was interpreted as being due to the removal of small amounts of molybdenum and zinc from the

sucrose, rather than to the removal of bios or other accessory growth substances

These references and others of a similar nature present an impressive argument for the inorganic salts, and it is not surprising that a number of the foregoing workers expressed doubts of the existence of accessory growth substances of organic nature in yeast decoctions or tissue extracts

On the other hand, there is evidence that ashing of tissue preparations destroyed the growth-promoting effect The writers (66, 126) found that ashing of active fractions obtained from veal infusion, liver, spleen, yeast and white potatoes caused a complete loss of the growth-promoting property Schopfer and Moser (139) in studying the factors in wheat germ for molds state that the mineral substance present in the ash of several extracts was not responsible for the growth-promoting effect Tatum, Peterson and Fred (150) ashed the Neuberg filtrate fraction in connection with their work on propionic acid bacteria and found that the ash did not produce the stimulative effect of the original extract Clark (17) found that ashing and wet combustion destroyed the growth factor for *Rhizobium* M Lwoff (81) reported that "active" iron compounds, as employed by Baudisch for *H influenzae*, were not effective as substitutes for hemin in supplying the needs of the trypanosome *Strigomonas fasciculata* The writers and their associates (62) were unable to demonstrate any growth-promoting effect when various amounts and combinations of inorganic salts, particularly those of the heavy metals, were substituted for active growth-factor preparations from tissues

Concerning the importance of the inorganic salts and particularly of the metals which act as catalysts in biological systems there can be no doubt whatsoever It is unfortunate that our knowledge of the mineral requirements of microorganisms is so incomplete that we are continually uncertain, when attempting cultivation in simplified media, whether the proper compounds or the proper amounts have been supplied However, this objection has been met by some workers who have employed the ash of biological materials which support growth

It has been common practice to ignore the traces (or perhaps larger amounts) of these compounds which are present as impurities with the amino acids, sugars and other ingredients used for synthetic media Glassware, metallic filters and other sources contribute an additional supply

Doubtless, if we knew more of the mineral requirements of the microorganisms our efforts to obtain satisfactory and rapid growth of the fastidious types in synthetic media would be more successful Aside from these important inorganic ingredients, however, recent work has revealed the significance of organic entities which are essential for the development of some of the more exacting bacteria, yeasts and molds It would appear, therefore, that the basic idea of searching for such organic compounds need not be altered, but that along with such endeavor there should be an alert recognition of the importance of the inorganic constituents

GROWTH-PROMOTING EFFECTS AND REMOVAL OF INHIBITING AGENCIES

Another explanation for the growth-promoting effects which follow the addition of tissue extracts to a simplified medium is that the added organic matter has combined with certain "toxic" or inhibitory substances present in the medium, thereby removing a harmful agent which previously restrained cell proliferation This suggestion was advanced by Fernbach (31) and Windisch (174) in the early discussions on the effect of bios on yeast growth, and it has since appeared from time to time in connection with the studies on bacteria Windisch in particular called attention to the presence of copper in distilled water and in media

It seems unnecessary to review here the many reports dealing with possible inhibitory effects of the varied components of culture media One example, taken from the more recent literature, will serve as illustration O'Meara and Macsween (106, 107) found some commercial peptones contained sufficient copper to inhibit growth in ordinary nutrient broth when the inoculum consisted of only small numbers of cells The addition of blood serum to the medium rendered it suitable for growth, presumably

by combining with or precipitating the copper Here is an excellent example of apparent growth-promoting or growth-stimulating effect following the addition of blood serum While the possibility of such effects must always be kept in mind, there now appears to be ample evidence that growth factor activity cannot be accounted for solely on this basis

GROWTH-PROMOTING EFFECTS RESULTING FROM CHANGES IN PHYSICAL PROPERTIES OF THE MEDIUM

In the attempts to develop suitable culture media for the more exacting microorganisms there is evidence that the physical character of a medium is not only important, but at times may be *the* factor determining suitability of the medium With respect to the study of growth-promoting factors, various investigators have attributed the beneficial effect of tissue extracts to changes produced in the physical character of the medium

Differences in hydrogen-ion concentration, surface tension, osmotic pressure, and the oxidation-reduction potential are among the more obvious alterations which may result from the addition of tissue extracts or other growth-factor preparations Of these various properties the importance of a suitable pH is well recognized, and the oxidation-reduction potentials of culture media and of developing cultures have received serious study Less attention has been paid to the other properties Since several publications have emphasized particularly the possible misinterpretations of growth-factor effects due to changes in the oxidation-reduction potentials of the culture medium, most of our discussion here will be concerned with this aspect of the problem, but it must be realized that the same principles apply to the other physical properties

There is now considerable evidence that bacteria can multiply only in media where the redox potential is within certain limits and that the limiting zone, whether broad or narrow varies with the individual organisms The favorable conditions for growth which are brought about by various procedures such as the addition of tissue extracts, large inocula, boiling of the medium, etc, have been attributed, at least in part, to the reduction of oxidized

substances or to the establishment of a suitable reduction potential in the medium

Wright (177, 178) called attention to inhibitory properties of the usual peptone-infusion media, particularly when seeded with small numbers of cells, and attributed this effect to constituents of peptone in the oxidized state Heating the peptone solution with meat, during the course of preparation of the medium, improved its growth-promoting properties, and Wright believed this effect was due to reduction of the peptone, or certain of its constituents, thereby removing the toxic action He also suggested that the inhibitory effect must be taken into account in experiments relating to accessory growth factors Dubos (23) reported the presence in peptone of substances which were bacteriostatic in the oxidized state Their bacteriostatic action could be overcome by the addition of thioglycollic acid

Allyn and Baldwin (3) have also emphasized the importance of the oxidation-reduction character of media in the initiation of growth A yeast-mannitol medium supported growth of *Rhizobium* when inoculated with small numbers of cells, while in a nitrate-mannitol medium no growth occurred unless very large inocula were used The yeast medium was more reducing in nature than the nitrate-mannitol medium The nitrate-mannitol medium permitted growth with similar small inocula after the addition of thioglycollic acid, powdered agar, or other reducing agents In this instance a synthetic medium, upon the addition of reducing agents, supported growth as readily as a yeast medium Thorne and Walker (156) found that the addition of reducing agents such as cysteine or thioglycollic acid increased growth and oxygen utilization of *Rhizobium* in media composed of highly purified ingredients (nitrate, sucrose, and inorganic salts) Cysteine brought about increases comparable to those induced by brown sugar, which has been said to contain appreciable quantities of accessory factors They found no evidence that root nodule bacteria require any complex, unidentified substances for their growth From these reports it is evident that a growth-promoting effect may be the result of adjustment of the oxidation-reduction potential from a less to a more favorable

region, or from the reduction of oxidized ("toxic") substances in the medium

The importance of CO_2 tension in the cultivation of bacteria has been stressed by many workers and has been well reviewed by Knight (52) The effect of other changes in the physical character of the medium has also been reported Hitchens (44) recommended the addition of 0 1 per cent agar to ordinary broth In the resulting semi-solid medium a number of the more fastidious types developed more luxuriantly than in broth or on ordinary solid agar slants Another interesting example has appeared in studies on methane fermentation Breden and Buswell (8) found that addition of shredded asbestos to a liquid medium provided a suitable background for development of the methane-producing types which appeared to require the presence of finely-divided material in suspension With the shredded asbestos in place of sewage sludge, subcultures could be carried through many transplants

While it is true that many of the studies on growth-promoting substances have ignored possible changes in oxidation-reduction potential and other physical characteristics of the medium, the growth-promoting effects observed probably are not due to physical changes In the study of *Lactobacillus delbrucki*, Snell, Tatum and Peterson (145) noted that the addition of potato extract lowered the oxidation-reduction potential of the basal medium and produced a growth-stimulating effect However, substitution for the potato extract of agents such as cysteine, cystine or thioglycollic acid, which lowered the potential in like amount, did not produce the stimulating effect Rahn and Hegarty (114) found that substances used to lower the redox potential failed to stimulate and at times even slightly retarded acid production by *Streptococcus lactis* Koser, Saunders and associates (62) found that changes in the physical properties of the test medium suggested by the foregoing reports did not produce the growth-promoting effect shown by extracts prepared from tissues

In studying *Streptothrix corallinus* in a synthetic medium plus tissue concentrates, Reader (115) found that alterations of the

surface tension of the fluid, within ordinary limits, did not affect the amount of growth and concluded that the growth-promoting activity of the added concentrates was not due to lowering of tension of the medium

Our evaluation of these conflicting viewpoints leads to a conclusion similar to that expressed in the previous discussion on the effects of inorganic salts There can be no doubt of the importance of the physical character of the medium Unfavorable levels of redox potential or other less well-recognized properties may prevent cell proliferation as effectively as unfavorable ranges of hydrogen-ion concentration In some cases an apparent growth-promoting effect may well have been due to the alteration of such conditions Unfortunately, we are still quite vague as to what many of the physical specifications should be and so the whole subject is left in a rather uncertain state It seems doubtful, however, that the growth-promoting effects of minute amounts of such compounds as thiamin, nicotinic acid, and β-alanine can be explained as due to a change in the physical properties of the medium

DEFINITE COMPOUNDS WHICH SHOW GROWTH-PROMOTING ACTIVITY

By way of summary, the compounds which have been substituted successfully for the complex mixtures of plant and animal tissue extracts are listed in table 1 Only those substances which seem to fill a fundamental and often specific need for cell proliferation are included With one exception, the chemical structure of all of these compounds is now definitely known

Hematin or hemin This iron-containing compound needs little comment here since it has been discussed in earlier reports It appears to have been the first of the so-called accessory substances for microorganisms to be definitely identified In addition to its important rôle in the cultivation of bacteria, it has also been shown to substitute for a component of blood in the cultivation of several trypanosomes

i-Inositol The inclusion of i-inositol in our list may be open to some question since by itself it is not sufficient for cell multipli-

cation, but the presence of one or more "cofactors" is necessary Furthermore it usually must be supplied in larger amounts than

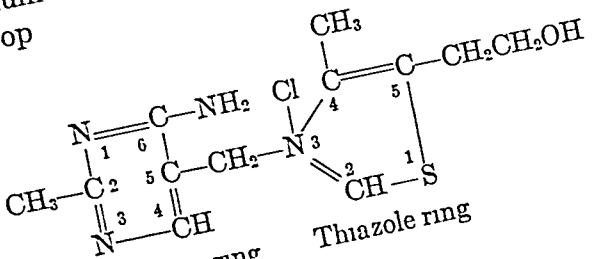
TABLE 1

Compounds which show growth-promoting activity

SUBSTANCE	ORGANISM	COFACTOR	REFERENCE
Hemin	*Hemophilus influenzae*	"V" factor	(20, 21, 153, 154, 155, 32)
i-Inositol	*Saccharomyces*	Other unknown substances	(25)
i-Inositol	*Nematospora gossypii*	Other unknown substances	(16, 58)
Thiamin	Molds	Other unknown substances	(129, 12, 130, 131, 58)
Thiamin	Propionic acid bacteria	Ether sol factor from yeast	(152)
Thiamin	Yeasts	i-Inositol	(172)
		"Pantothenic acid"	(170, 171)
		"Biotin"	(59)
Thiamin	*Staph aureus*	Nicotinic acid	(53)
		Nicotinamid	
Riboflavin	Lactic acid bacteria	Hydrolyzed casein	(175, 108)
		Ether sol factor from yeast	
Nicotinic acid and derivatives	*Staph aureus*	Thiamin	(53, 68)
Nicotinic acid and derivatives	*C diphtheriae*	β-Alanine	(95)
Nicotinic acid and derivatives	*Shigella paradysenteriae*	None	(61)
Cozymase	*Hemophilus parainfluenzae*		(76)
β-Alanine	*Saccharomyces*	Aspartic acid	(171, 87)
		Inositol	
		"Pantothenic acid"	
		Thiamin	
		Leucine	
β-Alanine	*C diphtheriae*	Nicotinic acid	(97, 63)

the other growth factors It has been included, nevertheless, because it represents one of the few instances where a definite compound has been identified as the active ingredient of a growth-

promoting preparation Its function in cell metabolism seems uncertain at the present time According to Eastcott (25) it is stored in the cells, since the inositol taken up by yeast from the culture medium can be quantitatively recovered by hydrolyzing the yeast crop



Pyrimidine ring Thiazole ring

Thiamin

Thiamin Studies of the two ring structures which compose the thiamin molecule have revealed an interesting diversity of requirements among those microorganisms for which this substance is effective as a growth factor In a few instances the *intact* thiamin molecule is required The pyrimidine and thiazole components when supplied as separate entities, in equivalent molar concentrations, are ineffective as a substitute for the whole molecule The two components cannot substitute for thiamin in the case of the protozoa *Strigomonas oncopelti* and *Glaucoma pyriformis* (82, 75) A similar need for the intact thiamin molecule has been reported for certain of the parasitic fungi, namely several species of *Phytophthora* (123a) and the basidiomycete *Ustilago scabiosae* (136) For the related *U violacea* thiamin can be replaced partially by the two components Evidently these microorganisms are unable to put together the two components to form the whole thiamin molecule or, in the case of *U violacea*, this synthesis is accomplished too slowly to permit normal development

Other types are somewhat less exacting in their requirements *Phycomyces blakesleeanus* requires both components of the thiamin molecule but not the intact molecule itself (138, 143, 124) This is also true of *P nitens* (124a), *Staphylococcus aureus* (54) and the flagellate protozoan *Polytomella caeca* (74) It appears that these

microorganisms are not able to synthesize either of the two component ring structures The molds *Absidia ramosa* and *Parasitella simplex* require both components for rapid development but can grow more slowly in the presence of the pyrimidine constituent alone (134) Apparently the thiazole is synthesized by these molds, but in an amount insufficient for normal growth

Still other microorganisms can develop as readily in the presence of only one of the components as when the whole thiamin molecule is supplied This is true of *Mucor ramannianus* (100) which needs only the thiazole constituent and also of the yeast, *Rhodotorula rubra* (135) and several higher fungi which require only the pyrimidine constituent (124a) There is some evidence that the component which is not required is synthesized by the organisms

In contrast to the foregoing are the many microorganisms which are able to develop in a synthetic medium devoid of thiamin While our knowledge of the physiology of these types is still quite incomplete, it appears probable that thiamin plays an important rôle in their metabolic processes Since in these cases neither thiamin nor its direct components are supplied, these organisms apparently possess the property of synthesizing the two ring structures from much simpler compounds

Derivatives of thiamin and its components There is evidence of a high degree of specificity in the chemical structure of the active compounds Thiochrome, an oxidation product of thiamin in which the nitrogen atom of the 6-amino group of the pyrimidine is linked to the 2-carbon atom of the thiazole ring, can substitute for thiamin only very imperfectly or not at all for growth of *Staphylococcus aureus* (54), *Phycomyces* (137) and *Rhodotorula rubra* (134) Also, a molecule similar to thiamin but lacking the β-hydroxyethyl group at the 5-position of the thiazole ring was inactive for *Staphylococcus* (54) Substitutions in various positions of the pyrimidine ring of the intact thiamin molecule greatly reduced or abolished the activity of thiamin (55a) The activity could be restored by addition of the normally substituted pyrimidine

Several substitution products of both the pyrimidine and the

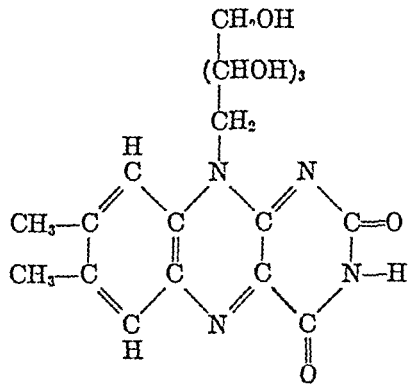
thiazole components have also been tested 2-Methyl-5-aminomethyl-6-aminopyrimidine[2] was active for *Staph aureus* in the presence of the thiazole component, while under the same conditions 2-methyl-5-hydroxymethyl-6-hydroxypyrimidine, 2-methyl-5-aminomethyl-6-hydroxypyrimidine and 2-hydroxy-4-aminopyrimidine (cystosine) were all inactive (54) When the 5-aminomethyl group in 2-methyl-5-aminomethyl-6-aminopyrimidine was replaced by a 5-thioformamidomethyl group, the compound retained activity, though in somewhat lessened degree, for *Staphylococcus* (54) and for *Phycomyces* (143) and was said to "substitute fully" for growth of *Rhodotorula rubra* (134) Upon substitution of a 5-bromomethyl for the 5-aminomethyl group, growth-promoting activity was retained for *Phycomyces* (in the presence of the thiazole component) (124) In a later report Knight and McIlwain (55a) used additional substituted pyrimidines and found that most of them were inactive for *Staph aureus* The groups attached to the ring which appear essential for activity are a methyl group at position 2, an amino group at position 6 and a methyl group substituted in certain ways at position 5 Thus at position 5, $-CH_2NH_2$, $-CH_2OH$ and $-CH_2NH$ CSH permitted growth, but $-CH_3$ and $-CH_2CO$ NH_2 were inactive Nucleic acid or hydrolysates of nucleic acid which supply pyrimidines were not effective for *Phycomyces* when substituted for the specific pyrimidine (124)

Substitutions in the thiazole component have demonstrated a similar high degree of specificity For growth of *Mucor ramannianus* 4-methyl thiazole, 4,5-dimethyl thiazole and 2-mercapto-4-methyl thiazole were all unable to take the place of the usual 4-methyl-5-hydroxyethyl thiazole (100) For growth of *Phycomyces*, Robbins and Kavanagh (124) found that a number of other thiazole derivatives were ineffective as substitutes for the usual component Likewise a number of sulphur-containing compounds such as methionine, glutathione, thioglycollic acid and others were ineffective For growth of *Staph aureus* Knight

[2] The designation of the pyrimidine derivatives has been changed in this article to conform with the usual system of numbering the positions in the pyrimidine ring

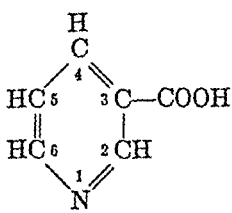
and McIlwain (55a) found other substituted thiazoles were either inactive or showed reduced activity

Judging from the relative effects on *Staph aureus* of the different substituted groups at position 5 of the pyrimidine, it appears probable that the pyrimidine and thiazole components are joined to form the intact thiamin molecule, rather than that the two components are used separately (55a) Certain observations of Hills (43a) on pyruvate metabolism by *Staph aureus* support this hypothesis, as does the work of Robbins and Kavanagh with *Phycomyces* (124)

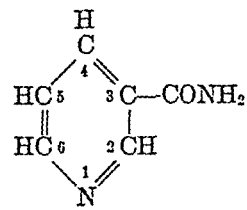


Riboflavin

Riboflavin Little information concerning riboflavin appears to be available aside from that given in the references previously listed Other related compounds possessing the isoalloxazine or alloxazine rings have not been available or their activity has not been tested in bacteriological work



Nicotinic Acid



Nicotinamide

Nicotinic acid and nicotinamide, coenzyme, cozymase The recent work of Warburg and of Euler and their associates demonstrated that nicotinamide is a constituent of the coenzyme[3] from horse blood and the chemically related cozymase[3] from yeast The nitrogen in the pyridine ring of nicotinamide is important in the transfer of hydrogen in biological oxidations It is of interest that in the case of *H parainfluenzae* (76), the pyridine nucleotide di- or tri-phosphate was required and nicotinic acid, nicotinamide, and adenylic acid (adenine + d-ribose + phosphoric acid) could not substitute as growth factors In the case of the staphylococcus, the diphtheria bacillus and the dysentery bacilli only the nicotinic acid or its amide was needed The comparative activity of nicotinic acid, nicotinamide and some related compounds has been studied in a few cases According to Mueller (95) the amide was about one-tenth as effective as the acid for the diphtheria bacillus In studies on the staphylococcus Knight (53) found the amide to be about five times more potent than nicotinic acid in the presence of appropriate amounts of thiamin Methyl nicotinate also was effective but development of cultures was slower Pyridine-3-nitrile was ineffective as such but was active after hydrolysis (yielding nicotinic acid) In a later publication Knight and McIlwain (55a) reported that the following compounds were all inactive coramine, pyridine-3-sulfonic acid, β-picoline, nicotine, trigonelline methyl sulphate, trigonelline chloride, isonicotinic acid, picolinic acid, quinolinic acid, 2,4-dimethylpyridine-3,5-dicarboxylic acid and 2,4,6-trimethylpyridine-3,5-dicarboxylic acid Landy (68) has reported that the two isomers of nicotinic acid (picolinic and isonicotinic acids) cannot replace nicotinic acid for the growth of *Staph aureus*, α- and γ-picoline were also ineffective Nicotinamide and the N-ethyl nicotinamide were both active but the N-diethyl compound was inactive Sodium and ammonium nicotinate were as effective as nicotinic acid for the growth of ester of the acid was slightly less active In studies on the dysentery bacilli Koser, Dorfman and Saun-

[3] Constituents of coenzyme and cozymase are adenine, nicotinamide, 2 molecules pentose and either 3 or 2 molecules, respectively, of phosphoric acid

ders (61) found the amide to be slightly more effective, but the difference between the amide and the acid was not marked In a more extended study of nicotinic acid derivatives Dorfman, Koser and Saunders (22a) showed that pyridine-3-sulfonic acid, trigonelline, 6-methyl-nicotinic acid, nipecotic acid, isonicotinic acid, β-acetylpyridine, β-picoline, and pyridine were devoid of growth-promoting activity The following substances favored good growth in the dilutions indicated nicotinic acid, nicotinamide, methyl nicotinate M $\times$ 10^{-7}, trigonelline amide, ethyl nicotinate, nicotinuric acid, ethyl nicotino-acetate M $\times$ 10^{-6}, nicotinic acid N-methyl amide M $\times$ 10^{-5}, nicotinonitrile M $\times$ 10^{-4} Picolinic acid and quinolinic acid showed activity at a dilution of M $\times$ 10^{-4} but there is a possibility that these two preparations may have been contaminated with traces of nicotinic acid

Beta-alanine The growth-promoting activity of this amino acid for some yeasts and the diphtheria bacillus is quite in contrast to the negative results secured with the ordinary α-alanine Many of the protein hydrolysates or basal synthetic media in which the diphtheria bacillus has failed to develop have contained α-alanine Addition of one microgram or less of β-alanine per cubic centimeter of medium fills some need for cell multiplication which is not supplied by the α-form This importance of a β-amino acid is of particular interest since in the past biologists and chemists have considered the α-amino acids as being the only ones of biological importance In view of the incomplete knowledge of the composition of proteins and other tissue extractives, it may well be that β-amino acids play a far more important rôle than has heretofore been recognized

The diphtheria bacillus is capable of obtaining β-alanine from naturally occurring *l*-carnosine but not from the *d*-form (Mueller, 96) Upon acid hydrolysis, both *d*- and *l*-carnosine yield equally active products

It might also be added that asparagine and aspartic acid which have been commonly used in synthetic media, can yield β-alanine and it is quite possible that many organisms capable of developing in the simpler synthetic media can bring about this change and

secure β-alanine from asparagine In other words, the need for β-alanine may be much more wide-spread among microorganisms in general than indicated by the results with some yeasts and the diphtheria bacillus, but many types may secure it from asparagine or other sources

Biotin Tentative empirical formula, $C_{11}H_{18}O_3N_2S$ This substance has been included in the list of growth factors although its structural formula is not yet known From the reports of Kogl and associates (57) it appears to be a definite entity It is an amphoteric substance and its methyl ester has been obtained in crystalline form The evidence thus far submitted seems to show that it is important in the cultivation of a number of microorganisms belonging to quite different groups and that very minute amounts of it exert a distinct growth-promoting effect

SYNTHESIS OF ACCESSORY GROWTH FACTORS MUTUAL INFLUENCES

It seems reasonable to believe that many of the growth factors listed in the foregoing section and others still unrecognized are required by microorganisms in general For many types there is no need to supply them as such, because the organisms presumably can synthesize them from simpler substances Here and there, however, we encounter a type which is unable to synthesize *one* of a number of required substances (e g , dysentery bacilli and nicotinic acid) When this one compound is supplied along with needed sources of nitrogen, energy and inorganic salts, rapid multiplication ensues Other organisms happen to be unable to manufacture *two* of these substances (e g , the staphylococcus with respect to thiamin and nicotinic acid) and do not develop unless both are supplied Either compound in suboptimum amount limits growth Again, an organism may be totally unable to synthesize one needed compound but can construct another required substance slowly—too slowly for normal growth Here one substance is essential and another serves to stimulate growth In a similar way, other more exacting microorganisms will doubtless be found to need an assortment of various substances which they themselves cannot produce Thus,

there are a number of interacting compounds and often the action of any one becomes evident only in the presence of the others It is obvious, too, that the building material which is available for the organism will doubtless vary from one situation to another so that the kind of "raw product" offered may often determine in large measure whether or not certain compounds can be synthesized

It is believed that the lack of synthetic abilities, with resulting "fastidiousness" of the organism, represents a loss of properties in connection with adaptation to a commensal or a parasitic mode of life and that it is not due to the acquisition of new growth requirements (52, 76) In nature those organisms which are unable to accomplish such syntheses must depend upon production of the required compounds by other types Many of the instances of growth stimulation of one type by another, seen on the ordinary laboratory media, can doubtless be explained on this basis

The familiar "satellite" phenomenon of Grassberger (41), who called attention to the increased size of colonies of *H influenzae* when growing in close proximity to colonies of staphylococci, has been often encountered with many other species A few instances associated with definite growth factors follow In the work on thiamin Muller and Schopfer (100) found a mold (*Mucor ramannianus*) which was incapable of synthesizing one component of the thiamin molecule and so was unable to develop unless this structure was supplied A yeast (*Rhodotorula rubra*) needed only the other thiamin component These organisms were capable of developing together in a simple medium, without any added thiamin, since each manufactured the particular component of the thiamin molecule needed by the other Another instance was reported by Kogl and Fries (58) with respect to *Polyporus adustus* and *Nematospora gossypii* Neither of these fungi was able to grow in a synthetic medium in pure culture, when inoculated together, however, they developed *Polyporus* requires thiamin which was supplied apparently by *Nematospora*, while the biotin requirement of *Nematospora* was supplied by *Polyporus* With the varied synthetic abilities of diverse organ-

isms and the many situations encountered in nature there would seem to be almost no limit to the number of such combinations

Other striking relationships have been reported between microorganisms and the higher plants Of particular interest are those concerning the fungi and orchids, and the relationships between the root-nodule bacteria and legume plants A review of this aspect of the problem has been given by Bonner (5) A fuller recognition of the limitations of synthetic abilities of organisms would doubtless help in no small degree in explaining some of the baffling symbiotic and other relationships so frequently encountered in nature The apparent inability of many of the pathogenic microorganisms to synthesize accessory growth factors, such as nicotinic acid and thiamin, seems highly significant in connection with their invasion of the tissues of the higher animals and plants where these substances may be found

FUNCTION OF THE ACCESSORY GROWTH FACTORS

What essential rôle is played in the physiological processes of microorganisms by the minute amounts of these growth substances? A consideration of the substances now known to possess growth-promoting properties shows that most of them enter into the structure of enzymes or coenzymes concerned with cell oxidations Thus, the pyrophosphoric ester of thiamin, thiamin diphosphate, functions as a cocarboxylase with a protein of yeast cells and in this enzyme system strongly promotes the decarboxylation of pyruvic acid, an important intermediate product in the dissimilation of glucose Nicotinamide is one of the components of the coenzyme of Warburg and of cozymase which plays an important rôle as a mediator in biological oxidation Riboflavin when combined with phosphoric acid and protein becomes the "yellow enzyme" of Warburg and Christian which, together with a second enzyme and the coenzyme, brings about the oxidation of hexose-monophosphoric acid ester, an important step in sugar oxidation

In these cases, the fulfillment of the "growth factor" requirements of one of the more fastidious microorganisms furnishes a portion of an enzyme or coenzyme molecule which the organism

itself cannot synthesize, but which it needs in order to carry on its metabolic processes Without the needed component the sequence of events in the respiratory chain is broken and cell multiplication is not possible Since these substances enter into a catalytic respiration system it becomes apparent why such minute amounts suffice

A similar function has been suggested for hemin (80, 73, 78) in relation to certain trypanosomes and *H influenzae* In the past, much attention has been centered on the peroxidase or catalase activity of X factor as a protective mechanism against toxic peroxides From the work of the Lwoffs, however, it appears reasonable that the need of the hemophilic microorganisms for hemin or X factor is connected with inability to synthesize the prosthetic group of a respiratory enzyme

In past years, attempts to cultivate the more exacting types in chemically definite media have considered for the most part only the question of structural material for the cell proteins and neglected the materials required for the building of enzyme systems or other special needs Rahn (113) has suggested that the vitamin-like substances might be needed for construction of certain special molecules in the cell, for example the genes Since these substances would enter into the structure of only a few molecules in the cell, therefore only very small quantities would presumably be required It is now apparent that the enzyme-coenzyme systems may be included among the cell constituents for which special structural material is needed

Of the definite compounds now associated with growth-factor activity for microorganisms, i-inositol and β-alanine have not been shown to be components of an enzyme system, insofar as the writers are aware The interpretation of their rôle in cellular metabolism must await further evidence In the meantime, it is an interesting thought that the demonstration of the important part which these substances play in development of certain microorganisms may give a clue to their occurrence in some enzyme-coenzyme systems whose composition is now unknown

The present knowledge of the growth factors, while fragmentary, permits a clearer idea of future lines of work which should

prove to be fruitful, and we are now better able to direct our efforts in solving the mysteries which still surround the growth requirements of many of the microorganisms If one component of a coenzyme, such as nicotinic acid for example, is needed by a microorganism, perhaps two, three, four or more components of this or other systems may be required by still more exacting pathogens, or by some of the more fastidious types important to agriculture or to the fermentation industries Following this line of reasoning, it might be assumed that some of the strictest parasites, which multiply only in the presence of living tissue or within living tissue cells, have lost a large measure of constructive ability in connection with their adaptation to such an abode Such organisms might conceivably be unable to put together a needed organic catalyst even when supplied with its several component parts and perhaps will be found to need the intact, preformed constituents of a whole system

SUMMARY

A number of attempts have been made to isolate the growth-promoting substances known to be widely distributed in animal and plant tissues In many instances identification of the growth substances has not yet been accomplished, though some progress has been made in their separation In other cases, however, several compounds of known chemical structure are now recognized as the active substances of tissue extracts These are hemin, i-inositol, thiamin, nicotinic acid and its amide, β-alanine, riboflavin and pyridine nucleotide phosphate (coenzyme or cozymase) With the exception of i-inositol, these compounds are needed only in very small amounts

The microorganisms for which one or more of these compounds must be supplied are *H influenzae* and related types, propionic and lactic acid bacteria, staphylococci, diphtheria bacillus, dysentery bacilli, certain of the true fungi including some of the yeasts, and certain protozoa On substitution of the required compounds for tissue extracts, it is now possible to cultivate a number of these types in synthetic media

Another substance, biotin, has been obtained in crystalline

form as the methyl ester Others have been obtained in a relatively pure state the "sporogenes vitamin," pantothenic acid, the "L" fraction for lactic acid bacteria

Microorganisms requiring the foregoing compounds are unable apparently to synthesize them There is increasing evidence that other less fastidious types are able to construct them from simpler substances The various constructive abilities of different organisms are significant with respect to symbiotic and other mutual relationships

With the exceptions of i-inositol and β-alanine, the accessory factors are known to enter into the structure of enzyme-coenzyme systems catalyzing oxidation processes

The growth-promoting effect of tissue extracts cannot be explained solely on the basis of the inorganic salt content or an alteration in the physical properties of the culture medium

It is significant that recent work has tended to show the close relationship between the nutrition and metabolism of microorganisms and the higher forms of plant and animal life

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THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

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The fibrinolytic activity of hemolytic streptococci is a term used to designate the capacity of broth cultures of *Streptococcus hemolyticus* of the *beta* type to transform the solid clot of normal human blood into a liquid state The rapid dissolution of human fibrin by hemolytic streptococci is dependent upon the presence in cultures of an extracellular enzymic substance which is excreted by the living organisms Reports in the literature evidence the fact that the phenomenon has special characteristics of bacteriological and immunological interest

The fact that the reaction involves a special kind of bacterial product acting upon a special kind of tissue substrate illustrates the particular qualities of streptococcal fibrinolysis The process

seems to differ from the catabolic action of proteolytic enzymes which reduce complex protein material to split products of relatively simple chemical composition Furthermore, a possible correlation with bacterial toxins, which are excreted extracellularly, remains uncertain in the present state of knowledge However, the phenomenon appears to belong to the types of reaction which include enzymes and toxins, and, for this reason, warrants consideration from a biological standpoint and also as a possible agency in the mechanism of infections due to hemolytic streptococci

It is the purpose of this article to review and to attempt to evaluate, when possible, the published reports concerning fibrinolytic action of streptococci and other bacteria The editors of *Bacteriological Reviews* have urged their authors "to distinguish between the essential detail and the isolated, vanishing particular" The fulfillment of these conditions is rendered particularly difficult in the present report because of the fact that the investigations have developed only in recent years From the published articles of numerous investigators, it is possible to define more clearly some phases of the reaction However, lines of study suggested by certain factors of streptococcal fibrinolysis have yielded results which have served to broaden the scope of inquiry Since these findings in many instances constitute new data, it is apparent that final conclusions cannot be drawn for the present All the reports, which this author has encountered, have been included for the purpose of bringing the subject matter up to date, even though the diversity of some individual researches and the fragmentary reports of others renders difficult a critical assay of some of the results

The terminology used in reference to some phases of the reaction is to some extent unsatisfactory Certain phrases are perhaps awkward or confusing However, at the present time, it would appear to be premature to offer a glossary of fixed expressions In the current state of knowledge it seems desirable to refer to the phenomenon in terms which emphasize certain special conditions, not for the purpose of imposing exact and restricting definitions, but rather to identify the reaction by specifying the particularly striking features

I MATERIALS AND METHODS OF EXPERIMENTAL PROCEDURES

The fibrinolytic reaction of cultures of hemolytic streptococci is readily demonstrable without any unusual precision of technique The occurrence of the phenomenon has been commonly encountered in tests with large numbers of strains of hemolytic streptococci However, laboratory conditions which may affect the results, have been noted by several observers Some of the conditioning factors assume importance only in certain types of technical procedure which will be referred to under appropriate headings Other experimental details, however, require more careful general consideration because they need to be taken into account in interpreting results They also indicate some of the biological factors which influence the production of fibrinolysin by the bacterial cells From the standpoint of critical analysis it is, therefore, advantageous to review, first, data concerning experimental procedures

One of the interesting aspects of the reaction concerns the source of the materials used in obtaining the lysis of fibrin by cultures For example, the characteristically rapid and complete liquefaction of fibrin is usually most strikingly demonstrable when, on the one hand, the bacterial constituent of the test consists of cultures of hemolytic streptococci derived from *man*, and on the other hand, the fibrin, which serves as substrate, is also obtained from *man* Furthermore, negative or inconclusive results are most frequently obtained when cultures isolated from animal sources are tested against human fibrin, or when cultures derived from human infections are tested against animal fibrin As will be shown later, the findings just mentioned are not absolute, but are dependent upon quantitative as well as qualitative factors

The experimental conditions under which the clot-dissolving property of cultures is most satisfactorily obtained consist in mixing the cultures with plasma or fibrinogen before inducing clot formation By this procedure the organisms and their products are disseminated within the body of the clot as it forms, thus affording maximum surface contact between the active bacterial agent and the fibrin substrate

It has been noted by several observers (Tillett and Garner (65), Hadfield, Magee, and Perry (19), Madison (31), Dack, Woolpert and Hoyne, (4), Schmidt (57), and others) that the time required for dissolution of normal human fibrin by active cultures varies from a few minutes with some strains to a partial effect exerted by other strains during twenty-four hours' incubation 0 5 cc of broth culture plus 0 2 cc of a 1 to 5 dilution of plasma have been frequently used in tests and have given satisfactory results Some investigators have, for convenience, employed approximate fractions or multiples of the ratio given above The evidence is clear that the differences in speed and completeness of fibrin dissolution exerted by strains is dependent upon quantitative differences in the amount of fibrinolysin excreted by the cultures Consequently, it is obvious that the demonstration of the occurrence of lytic action by strains, as well as the degree of potency, may be conditioned by the quantities of reagents selected for use

The importance of the quantitative factor is also indicated by the report of Madison (33) concerning results obtained following the concentrations of fibrinolysin contained in cultures of several strains (The methods of concentration will be described later) Madison found that among 123 strains only 17 per cent were considered to be actively fibrinolytic when the tests were made with 0 5 cc of broth culture However, the percentage of demonstrably positive strains was raised to 35 per cent when cultural material which had been concentrated approximately twentyfold was employed

The quantitative production of fibrinolytic substances by strains is also related to the cultural conditions under which the tests are made Madison and Taranik (39) compared the curve of bacterial growth with the production of fibrinolysin, and found that "test tube proliferation" of the bacterial cells paralleled the rate of production of the lytic enzyme Using quantitative titration, they were able to demonsrate lytic activity with cultures after a few hours' incubation and they also noted that the production of fibrinolysin was maximum when the phase of growth was nearest the peak, which was reached after approximately 12 to 14 hours After this point in multiplication had

been reached, the production of fibrinolysin was markedly retarded, although some of the cultures retained maximum activity after twenty-four hours' incubation The experiments did not indicate with certainty whether or not enzyme production requires cell division

Without making quantitative titrations, Tillett and Garner (65) noted gradual deterioration of lytic activity in cultures which were kept in the incubator for several days Decrease in potency was delayed, but not entirely arrested, by ice-box temperatures Schmidt (57) found cultures five to six days old to be active, but he did not report measurements of activity

It may be seen from the findings just cited that fibrinolysis by active cultures may be demonstrated within wide ranges with respect to age of cultures However, it is also brought out that greatest activity is determined both by the abundance of growth of the strains and by the time in the phase of growth at which tests are made With bacterial strains of high fibrinolytic activity the qualitative demonstration of lysis requires no special attention to cultural details However, with strains which elaborate relatively small amounts of fibrinolysin, experience has shown that the extent of multiplication of streptococci—which may be limited in unfavorable media—and the age of the culture may be important factors in determining the results of fibrinolytic tests with individual strains

There is, also, suggestive evidence that additional elements in culture media, in the nature of accessory substances, may promote or retard the yield of fibrinolysin by streptococci There are no published reports dealing with this point However, the author has noted that when selected strains were cultivated simultaneously in samples of culture media containing different ingredients, the lytic potency of individual strains varied, even though the amounts of growth seemed to be comparable in the different kinds of media When one considers by analogy the effect of culture media on the production by other bacteria of products such as toxins, it seems likely that the specific stimulation or impairment of the elaboration of fibrinolysin may be subject to conditions of the same order

Whether variations in the potency of cultures depends upon

differences in the number of individual cells of the culture population, which excrete fibrinolysin, or is referable to the amount of the enzyme produced equally, in any single culture, by all the cells, has not been studied The problem is common to the broader question concerning bacterial adaptation and selection, which, with respect to enzymes, has been discussed by Yudkin (84), who has considered the types of substances responsible for the increase in enzyme content of microorganisms

According to the classification of bacterial enzymes employed by Karstrom (25), the fibrinolysin seems to belong to the group designated as "constitutive" enzymes, which do not require the presence of substrate for the production of the enzyme, as opposed to "adaptive" enzymes which are formed only in the presence of the specific substrate No study has been made of the effect of the introduction of fibrin into cultures on the yield of fibrinolysin by the bacterial cells *In vivo*, the possibility that the fibrin of inflammatory exudate might promote the production of fibrinolysin by the infecting organism is suggested by the occurrence of highly potent fibrinolytic strains in widespread infections

Even though information concerning the effect of environment in the production of fibrinolysin is limited, it has been the common observation of many investigators that whereas many strains during artificial cultivation retain, as a constant property, the fibrinolytic potency exhibited in the initial tests, other strains have not maintained a uniform degree of lytic activity after repeated transplantations For example, Hadfield, Magee, and Perry (19) observed that, after ten to thirty subcultures, some of their strains were decreasing in lytic potency Of eleven active strains, they noted that six retained the same degree of activity during the period of study

Observations also indicate that strains, with which the rate of reaction has slowed down, may be restored again to highly active ones both *in vitro* and *in vivo* The factors which influence the yield of fibrinolysin by individual strains appear to be, in part, inherent in the bacterial cells and also to be related to the environments in which the organisms are kept viable This

subject will be considered again further on It is mentioned at this point to illustrate the fact that constancy in the yield of fibrinolysin has not been found to be a fixed attribute of all strains of hemolytic streptococci during periods of artificial cultivation

The cultural factors which afford the most favorable basis upon which to make observations require (*a*) Abundant growth (*b*) Use of culture at time of maximum growth (*c*) Use of culture media favorable for yield of fibrinolysin (possible influence of factors accessory to nutrition is suggested) It is important, also, to differentiate, in single tests on individual cultures, between strains that may, through prolonged laboratory cultivation or environmental circumstances, have become weakened in fibrinolysin production and other strains that are actually devoid of the property

In the usual performance of the test, the plasma from the blood of *normal* human beings is regularly employed However, the plasma-clots of different, apparently normal individuals may vary in susceptibility to lysis For example, Tillett, Edwards, and Garner (66) noted that among thirty normal adults the plasma-clots from the blood of thirteen were liquefied within fifteen minutes, whereas the fibrin from eight others required from one to four hours before lysis was complete, even when a highly potent strain of hemolytic streptococcus was used in the tests The dissolution time for the remaining nine normal persons ranged between fifteen minutes and one hour

In addition to the clot available in whole oxalated plasma, fibrinogen and thrombin chemically isolated from blood have also served as a source of fibrin (65) The fibrin formed by combining fibrinogen and thrombin in the presence of active cultures has been found to liquefy at a greater speed and with smaller amounts of culture than does the clot of whole plasma The probable explanations of the difference in speed of reaction between the substrates of whole plasma-clots and of fibrinogen-thrombin clots will be discussed in relation to immunological studies From the standpoint of experimental procedure, the greater sensitivity of the fibrinogen-thrombin material has been

found to be useful in certain studies However, it should be noted that additional complications may be introduced with the fibrinogen-thrombin technique Investigators studying problems of blood coagulation have observed that when the fibrin, formed by relatively pure fibrinogen and thrombin, is allowed to stand for several hours, spontaneous lysis may occur with some preparations Whether or not the spontaneous autolytic process may be catalyzed by the streptococcal fibrinolytic enzyme has not been studied No information is available by which the end products of the autolytic and fibrinolytic actions may be compared However, the possible effect of spontaneous lysis in fibrinolytic tests involving several hours' incubation may condition the evaluation of results obtained with fibrinogen-thrombin preparations, if the time required for lysis extends to several hours

A final technical consideration concerns the influence of spontaneous retraction of the clot on the reading of the results of fibrinolytic tests When active fibrinolysis occurs under the usual favorable conditions, the process is characteristic and the end point of the reaction is clearly defined The quantities of materials employed in the usual test are such that, when coagulation occurs, the tube may be inverted without disturbing the position of the clot at the bottom However, when the tubes are allowed to stand for a period of time, retraction of the clot may occur regardless of the presence or absence of bacterial cultures The factors which determine the retractility of blood clots appear to be unknown Consequently, neither the speed nor the degree of retraction is controlled in fibrinolytic tests When the clot remains attached to the inner wall of the tube, it seems to occupy most of the space up to the top of the fluid level and it is saturated with liquid Under these conditions, the reading of negative fibrinolysis is definite However, when the clot is released from the sides of the tube, it settles to the bottom and may progressively shrink in size depending upon the degree to which the fluid contained within the interstices of the clot is squeezed out An appearance comparable to the latter

incident may occur in fibrinolytic tests In the experience of the writer, it may be difficult to differentiate weakly acting strains which have induced partial lysis after prolonged incubation from nonfibrinolytic strains in the tests of which a considerable degree of spontaneous retraction has occurred without lysis Consequently some degree of reservation is indicated in the exact classification of cultures when the results are not clearly defined It seems likely that a correct interpretation of some of the doubtful tests requires a method more accurate than the visual estimation of lysis on the basis of the size or shape of the ball of fibrin

In performing the tests, the greatest number of observations have been made by incubating the preparations in the water bath at 37°C Reports have indicated that incubation at higher temperatures may be preferable Hadfield, Magee, and Perry (19) allowed the tubes to stand at 37°C until coagulation had occurred Following clot formation, 52°C was used They believed that lysis was hastened at the higher temperature In some of his experiments, Schmidt (57) considered that more satisfactory results were obtained at 45 than at 37°C Sherman and Niven (59) have advocated incubation at 53°C , after coagulation has occurred at room temperature They pointed out that this procedure eliminated the growth of the organisms during the test period so that the result of the test was dependent upon the amount of preformed fibrinolysin They noted that the duration of the tests could be shortened, since, if no lysis occurred in four hours, the result was not altered by prolonged incubation Garner and Tillett (15) found that the reaction proceeded at a slower rate at room temperature than at 37°C , and that lysis was even more retarded at ice box temperature

In summarizing the data concerning the materials and methods, emphasis has been placed upon factors which may affect the results obtained in fibrinolytic tests Inasmuch as the phenomenon is readily demonstrable, the possible importance of some of the conditions may appear to have been unduly stressed However, a review of some of the technical details

indicates the possible significance of experimental procedures in interpreting the results to be reported, in some of which discrepancies may be referable to materials and methods

II THE TYPES AND KINDS OF BACTERIA, PARTICULARLY STREPTOCOCCI, WHICH POSSESS FIBRINOLYTIC ACTIVITY

The first positive tests of fibrinolytic activity were obtained with strains of hemolytic streptococci derived from patients suffering from acute illnesses Additional information concerning the fibrin-dissolving action among many strains of streptococci has accumulated from the published reports of several investigators The incidence of the fibrinolytic property has been considered in relation to certain individual and group characteristics of the organisms and also to other biological reactions of streptococci Other species of bacteria have also been tested for the presence of lytic properties Although in most instances the results have been negative,certain interesting findings have been reported

a Streptococcus hemolyticus of the *beta* type

The first series of articles to be summarized under this heading deal with the results of fibrinolytic tests performed with hemolytic streptococci which were described by the authors as being associated with infections of varied clinical manifestations and degrees of severity The details of the association have not been given in every instance nor is the correlation between the culture tested in the laboratory and the etiological status clearly established with many of the strains However, the findings illustrate the occurrence of fibrinolytic properties among human pathogenic strains

Tillett and Garner (65) tested the fibrinolytic activity of twenty-eight strains from different conditions including septicemia, acute tonsillitis, scarlet fever, erysipelas, empyema, and cellulitis All of the strains were actively fibrinolytic *Result* 28 strains, 28 positive

Hadfield, Magee, and Perry (19) reported the results obtained with twenty-nine strains that were derived from cases of moderate and severe scarlet fever, fatal septicemias, puerperal sepsis, peritonitis, tonsil-

litis, and rheumatic fever The strains all caused lysis with varying degrees of potency and completeness during the test period Five of them were highly active, six were somewhat less active, and eighteen produced slow or partial lysis The highly active ones were from the severe cases *Result* 29 strains, 29 positive, with 11 highly active and 18 weakly lytic

Madison (31) recorded the results obtained with thirty-two strains from "internal human tissues" and 123 from "superficial human tissues" The first group consisted of strains from cases of pneumonia, septicemia, empyema, and meningitis Thirty of these were fibrinolytic Of the 123 strains in the second group, which came from erysipelas, furunculosis, fistula, sore throat, sinusitis, and acute gastritis, only twenty-one were fibrinolytic Five strains from erysipelas were highly potent *Result* 1st group, 32 strains, 30 positive (94 per cent) 2nd group, 123 strains, 21 positive (17 per cent)

Morales-Otero and Pomales-Lebron (43 to 45) in separate communications cite their results with thirty-three, fifteen, and forty-eight strains, respectively The first and third groups were derived from a variety of disease sources Of these eighty-one strains, seventy-nine exhibited the capacity to dissolve fibrin (One of the negative strains came from a patient convalescent from scarlet fever, the other from a case of lymphangitis) The group of fifteen strains were obtained from cases of recurrent tropical lymphangitis Two of the strains were described by the authors as effecting incomplete hemolysis and appear not to have been of the beta type The remaining thirteen strains were fibrinolytic *Result* 94 strains, 92 positive (98 per cent)

Hare and Colebrook (20), in one of their articles concerning infections due to hemolytic streptococci in parturient women, described the biological characteristics of a large number of strains Of fifty-six derived from cases of puerperal infection, fifty-five were actively fibrinolytic From eleven of the cases which had low-grade fever during puerperium, the strains in three instances were fibrinolytic In some of these mild cases the authors considered that the fever was of uncertain origin *Result* 56 strains from puerperal fever, 55 positive (98 per cent) 11 strains from mild febrile puerperium, 3 positive (27 per cent)

Dack, Woolpert, and Hoyne observed the lytic action of 303 strains from scarlet fever Twenty-five of them were derived from infected mastoids, and were all fibrinolytic Of the remaining 278 strains, only twenty-eight caused lysis of fibrin *Result* 25 strains from scarlet

fever complicated by mastoiditis, 25 positive 278 strains from scarlet fever, 28 positive

Fraser and Madison (12) tested sixty strains from scarlet fever and found them all to be fibrinolytic The highest potency was most frequent in the strains from severe cases *Result* 60 strains from scarlet fever, 60 positive

Tillett (67) reported the results obtained with 157 strains Of these, 140 were grouped, according to the source, into those from septicemia, acute suppurative diseases, (such as meningitis, peritonitis, empyema, mastoiditis, etc), erysipelas, acute tonsillitis with and without rheumatic fever or nephritis, and a single additional group including chronic disorders and normal carriers In these observations, the tests were made with the first subculture of the organisms after isolation from the patient Of the 140 strains of definite etiological significance, 139 were fibrinolytic An additional group of seventeen human pathogenic strains, obtained from other laboratories, were found to be actively lytic *Result* 157 strains from various disease sources, 154 positive (98 per cent)

Kodama (26) studied the biological properties of a large number of strains Of 130 strains recently isolated from human infections and from the throats of normal people, 128 were fibrinolytic *Result* 130 strains, 128 positive (98 per cent)

Stewart (62) observed the lytic activity of 211 strains which produced soluble hemolysin Of these, 146 were from surgical sources including puerperal infection, forty-five were from scarlet fever, and twenty from removed tonsils One hundred and eighty-six of the total were classified as fibrinolytic Of the twenty-five negative strains, sixteen were from surgical sources, seven from scarlet fever, and two from removed tonsils The negative strains were tested on the first subculture *Result* 211 strains from various sources, 186 positive (88 per cent)

Evans (11) in a report on the properties of *Streptococcus pyogenes* cited the fibrinolytic properties of thirty-three strains Thirty-two were fibrinolytic In a subsequent article on *Streptococcus scarlatinae*, thirteen strains were tested The average potency of the strains was not great, and four were found to be negative Evans designated as *Streptococcus scarlatinae* strains which exhibited certain selective sugar fermentations, the most important of which was inability to ferment salicin *Result* 33 strains of *Streptococcus pyogenes*, 32 positive (97 per cent) 13 strains of *Streptococcus scarlatinae*, 9 positive (69 per cent)

Tunnicliff (71) studied, among several groups of streptococci, the occurrence of lysis by nineteen which were of the hemolytic type They were isolated from scarlet fever, erysipelas, septic sore throat, endocarditis, and septicemia All were actively fibrinolytic *Result* 19 strains, 19 positive

Summarizing the findings just given, the figures are Total number of strains, 1299, of which 899 (69 per cent) were actively fibrinolytic

In the greatest number of the reports, however, the incidence of fibrinolytic activity by the pathogenic strains was greater than 90 per cent Madison (31) in the tests with strains described as obtained from superficial human tissues, and Dack, Woolpert, and Hoyne (4) in their scarlet fever strains reported the lowest incidence (17 and 16 per cent respectively) of lytic properties It may be noted that Evans (11) also considered *Streptococcus scarlatinae* to be less actively fibrinolytic than the *Strep pyogenes* group All of the reports with respect to *Strep scarlatinae* strains have, however, not been consistent Dack and co-authors considered the latter strains from severe cases to be highly potent

The findings have demonstrated that strains from suppurative and invasive types of infection are, with few exceptions, not only regularly possessed of fibrinolytic properties, but are also usually the most potent in causing lysis of fibrin For example, cultures derived from cases of septicemia, peritonitis, meningitis, or infections of the throat (acute tonsillitis, scarlet fever) where the organisms have invaded beyond the local pharyngeal tissues, constitute the strains which elaborate fibrinolysin in considerable quantity The findings with cultures from minor infections and perhaps with some *Strep scarlatinae* strains, indicate either the absence of lytic properties or that the production of fibrinolysin is characteristically impaired during laboratory cultivation The suggestion, implied in these results, of a possible association between lytic activity and pathogenicity will be discussed later

The occurrence of fibrinolytic properties in strains derived from normal persons has been studied less extensively In twenty-five strains isolated from the throats of patients with various chronic disorders and from normal persons, Tillett (67) found the

incidence of weakly lytic strains to be greater than that of highly active ones More detailed findings with cultures from normal throats will be given in association with the studies of relations to the Lancefield groups

From the standpoint of the classification of hemolytic streptococci on the basis of biological, biochemical and serological reactions, the admirable and detailed review of Sherman (58) includes data concerning fibrinolytic activity of strains in relation to other findings It would be repetitious to record here the reports which he has analyzed Consequently, the reader is referred to Dr Sherman's article for comprehensive data

The fundamental observations of Lancefield (28) concerning the serological classification of hemolytic streptococci has had such wide and important application in the orientation of this species of organisms that it is of paramount importance in the study of strains Sherman has brought together various findings concerning streptococci under the Lancefield groupings Consequently the results given here will be limited to the fibrinolytic activity of strains with respect to the Lancefield classification

b Relation to Lancefield serological classification

Group A hemolytic streptococci have come to be recognized as the group characteristically responsible for acute infections in man The fibrinolytic activity of strains identified serologically as belonging to Group A has been described in several articles with uniform results Hare (21) reported on sixty-three strains from the nose and throat of normal persons, and found sixty-two possessed fibrinolytic properties Kodama tested 160 Group A strains from cases of infections, from normal persons, and from stock cultures, and 157 were fibrinolytic Davis and Guzdar found each of twenty-eight Group A strains from normal throats to possess lytic properties Sherman and Niven reported four out of five strains which dissolved fibrin They cite one strain, originally isolated from a case of epidemic sore throat which belonged to Group A but was nonfibrinolytic when tested Hare and Maxted isolated ten Group A strains from the stools of patients with scarlet fever, each culture was active against fibrin

Seegal, Heller, and Jablonowitz recovered from monkeys nineteen Group A strains and found them fibrinolytic. Of the 285 strains identified as belonging to Group A by the investigators who tested them against fibrin, 280 (98 per cent) possessed fibrinolytic activity. Madison (32) suggested "a possible genetic linkage between these two specific bacterial characters." From observations upon 189 strains, he reported that the titre of fibrinolysin and the titre of Group A carbohydrate as determined by the ring test were closely correlated. However, as will be described in the reports which follow, strains other than Group A have been found to be fibrinolytic.

Groups B, D, E, F, and H. Without extending the details, strains belonging to these groups have been found to be negative by Hare, Hare and Maxted, Kodama, Sherman and Niven, and Seegal, Heller, and Jablonowitz. The reports include forty-one strains of Group B, fifty-four of Group D, a small number of Group E, eighteen of Group F and ten of Group H. Sherman and Niven have recorded some of their results as ±, indicating that possibly a slowly acting lysis may have occurred with some of the strains. The source of all of the strains was the throat, stools, or vagina of normal persons, or milk.

Group C. Among fifty-seven strains derived by several investigators (21, 26, 6, 45, 59) from throat, or vagina of normal persons or throat of monkeys (56), fifty-four were found to be fibrinolytic. Of eleven strains isolated from milk (59), none was fibrinolytic. In the biochemical tests of the strains isolated from normal persons, trehalose was fermented but not sorbitol. In the milk strains, Sherman and Niven observed that trehalose was unaffected, but sorbitol was fermented. Sherman has suggested, therefore, on the basis of this difference the terms "Human Group C" and "Animal Pyogenes Group C." It is interesting to note that many of the strains belonging to the "Human Group C" are fibrinolytic, but that the "Animal Group C" are negative. The strains belonging to Group C have only rarely (21, 58) been reported, up to the present time, as occurring in infections in man. They constitute, therefore, a group of fibrinolytic streptococci, which have not been considered significant in human infections,

although Hare refers to two strains originally isolated from cases of erysipelas

Reich has described the transformation of a strain of hemolytic streptococcus, Group A, which by prolonged and repeated passage through rabbits lost the original serological classification and gave positive precipitin reactions first with Group C antiserum and then with Group E antiserum Coincidentally the fibrinolytic activity was also lost When, however, the strain was cultivated in repeated subcultures in broth, the original Group A reaction returned and fibrinolysis was again demonstrable Gay and Clark (17), on the contrary, reported that a human strain "H", which had been passed through rabbits for nineteen years, belonged, at present, to Group A, and was capable of liquefying fibrin Data with regard to the loss by a strain of fibrinolytic activity coincident with change in serological type are limited to the report of Reich It is apparent that confirmation by the use of a large number of strains is necessary before the suggestive finding is established

Group G Of seventy-nine Group G strains, derived like the Group C strains from the throat, vagina, or stools of normal persons, seventy were fibrinolytic (21, 22, 26, 6, 45, 56)

From the data with respect to serological classification, strains belonging to Groups A, C, and G have been found to be fibrinolytic Strains belonging to Groups B, D, E, F, and H have proved to be negative Reports of a considerable number of other strains which are nonfibrinolytic will be reported in connection with animal strains In these latter strains, however, the serological classification was not made

Using the Lancefield classification for the identification of human strains both from patients and normal persons, the combined tests of serology and fibrinolysis demonstrate a correlation in 98 per cent of the tests with Group A strains In combination with the observations made with strains derived from active infection but not classified serologically, the similarity of the two groups of findings is apparent

As an arbitrary test for the separation of human pathogenic strains from innocuous ones, the determination of fibrinolysis

is a helpful procedure but is not necessarily conclusive in every instance The large proportion of nonfibrinolytic strains among the serological groups has been found in Groups B, D, E, and F, which on the basis of previous experience with immunological and biochemical tests have been classified as nonpathogenic for man Fry has reported three fatal cases of infection due to Group B hemolytic streptococci The strains were without fibrinolytic activity Fry described the special characteristics of the pathological anatomy which differed from the usual changes observed in fatal cases of hemolytic streptococcus infection, and discussed the possible significance of the disease picture from the standpoint of the qualities of the infecting organism

In addition to the non-fibrinolytic Group B strains which caused infection in Fry's cases, limitations on the evaluation of negative strains as non-pathogenic are also exemplified by a few other exceptional strains which have possessed definite etiological significance in active infection and which have been tested under advantageous laboratory conditions but did not exhibit lytic properties

From the standpoint of the interpretation of positive fibrinolytic tests as indicative of pathogenicity, restrictions in the significance of the results are based on reports that strains belonging to Groups C and G are only occasionally significant in human infections but are frequently fibrinolytic It is interesting to note, however, that the fibrinolytic strains of Groups C and G have usually been derived from human sources Sherman and Niven are of the opinion that some strains of various hemolytic species may induce the slow lysis of fibrin

In contrast to the dissolving action of human pathogenic strains, Tillett and Garner reported that hemolytic streptococci from animal sources were usually incapable of liquefying the fibrin of human blood These findings have been extended in several reports, although serological classification was not regularly reported Since the factors pertaining to animal strains concern the source of the fibrin substrate as well as the origin and biological characteristics of the cultures, a consideration of this interesting phase of the subject is given in Section IV The present

section continues with results obtained with other kinds of streptococci and other species of bacteria commonly associated with man

c Streptococcus viridans

Of this variety of streptococci, Tillett and Garner tested six strains and found each to be nonfibrinolytic Madison (33) reported thirty-three strains as negative in fibrin-dissolving tests The same author, even after using methods of concentrating fibrinolytic material, was unable to obtain lysis with green streptococci Stewart recorded that thirty-three strains belonging either to the *Strep viridans* or *Strep anhemolyticus* type, were not active in the liquefaction of clot Schmidt obtained no lysis with green streptococci Laca and Porzecanski found strains of *Streptococcus viridans*, *Streptococcus fecalis*, and *Enterococcus* to be nonfibrinolytic Tunnicliff (71) stated that strains of *Streptococcus viridans* were nonfibrinolytic but that some of them inhibited clot formation

Neter and Witebsky (48) subsequently presented a series of reports on the fibrinolytic and anticoagulating action of several species of bacteria Although the immediate purpose of this review is not concerned with the so-called anticoagulating action of organisms, the findings which are related to fibrinolysis warrant consideration Neter and Witebsky reported that, when the bacteria were cultivated in 2 per cent glucose broth, some strains of the following species were fibrinolytic, *Streptococcus hemolyticus*, *Streptococcus viridans*, *Enterococcus*, *Pneumococcus*, *B coli*, *B lactis aerogenes*, *B friedlanderi*, *B pyocyaneus*, and *B proteus* They concluded that "fibrinolysin production is not limited to hemolytic streptococci alone, if, for instance, the sugar content of the culture media is increased " If this reviewer understands the article correctly, tests for fibrinolytic activity were considered positive if clot formation failed to occur when $CaCl_2$ was added to the mixtures of plasma and culture Witebsky and Neter (81) also described what they considered to be the properties of two different fibrinolysins produced by streptococci One of these had the following characteristics It developed when the organisms were grown in 2 per cent glucose broth, it inhibited

clot formation, it was effective in both human and animal plasma, it acted only in an undiluted state, it was thermostable, and it was not neutralized by antistreptococcus sera The other fibrinolysin was produced in 0 05 per cent glucose broth, it acted only upon human fibrin-clot, it was effective in high dilutions, it was thermostable, and was neutralized by antistreptococcus sera

Witebsky and Neter stated that, when cultures of *Streptococcus viridans*, *Enterococcus*, or *Pneumococcus* were cultivated in 2 per cent glucose broth, fibrinolysin developed like that present in cultures of hemolytic streptococci also grown in 2 per cent glucose broth

The inhibiting effect on clot formation exerted by cultures of streptococci had previously been noted by Dennis and Berberian and by Tunnicliff In the latter studies, the culture medium of choice was, respectively, 2 per cent dextrose broth (9) and 1 per cent meat extract broth with 1 per cent dextrose (71)

Dart reported a confirmation of the findings of Neter and Witebsky with respect to fibrinolysin and anticoagulant (second fibrinolysin) if hemolytic streptococci were cultivated in 0 4 per cent dextrose broth The fibrinolysin was obtained from cultures by precipitation with alcohol according to the method described by Garner and Tillett, the anticoagulant factor was recovered from the supernatant fluid by evaporation, it resisted heating at 100°C for 30 minutes

Dennis and Adham in a further study of the anticlotting factor of dextrose-broth cultures of streptococci described it as being soluble in 75 per cent alcohol, absolute alcohol, and ether, it was dialyzable, it gave a strongly positive Kelling's test for lactic acid They concluded that the anticoagulant was primarily lactic acid The anticlotting constituent seldom occurred with cultures grown in media having less than 0 4 per cent dextrose, and the authors considered the anticlotting action to be more closely correlated with the total acid content of the cultures than with pH

Tillett (69) studied the anticoagulating effect and the fibrinolytic activity of strains of *Streptococcus hemolyticus*, *Streptococcus viridans*, and *Pneumococcus* The cultures were cultivated in 0 05, 1 0, and 2 per cent dextrose broth With respect to the anti-

clotting effect, he found that when the ultimate pH of the 1 0 or 2 0 per cent dextrose-broth cultures was below 5 0, coagulation of plasma was inhibited, when the pH of the cultures was above 5 0, clotting occurred in all the tests but fibrinolysis was effected only with strains of *Streptococcus hemolyticus* With uninoculated sterile broth of varying hydrogen ion concentrations, the effects on the coagulation of plasma paralleled the findings obtained with cultures of the same pH Furthermore, when the high degree of acidity (pH 4 4 to 4 9) produced in dextrose broth cultures was altered by the addition of NaOH to pH 6 0 to 7 0, coagulation occurred When cultures in 0 05 per cent dextrose broth (pH 6 5 to 7 0) were acidified to below pH 5 0, coagulation was inhibited In studies on the physiology of blood coagulation, the lower limit of pH at which fibrin is formed is placed at 5 6 to 6 0 It is also interesting to note that the anticoagulative action of organic acids, including lactic acid, has been described (80) Tunnicliff and Hammond (72) in continuing a study of the anticlotting action of *Streptococcus viridans* found that the smooth form, which prevented coagulation, lowered the pH of 1 per cent dextrose broth to 4 4–4 8, cultures of rough forms, however, which did not inhibit coagulation, reached a pH of 5 2–6 0

From a consideration of the data concerning the anticlotting action of various bacterial species, it seems probable that the effect depends to a considerable degree on the action on oxalated plasma of the products of the hydrolysis of sugar by the organism, or on pH, or on both of these factors Furthermore, from an analysis of the findings with respect to organisms other than hemolytic streptococci, it appears that the action designated as fibrinolysis by *Streptococcus viridans*, pneumococci, and other bacterial species, is not due to a lytic agent comparable to the fibrin-dissolving substance of hemolytic streptococci

d Other streptococci, Dissociants

Pseudo-hemolytic streptococci This term has been frequently employed by English investigators in designating strains which differ from other hemolytic streptococci on the basis of negative tests for "soluble hemolysin" Hare and Colebrook describe the

results of fibrinolytic tests with thirty-four such strains None of them caused lysis of fibrin Twenty-seven of the strains were from pregnant women who had afebrile puerperium Seven came from puerperal cases with mild fever Stewart (62) found that twenty-seven strains of the pseudo-hemolytic variety were nonfibrinolytic

Streptococcus anhemolyticus Only a few strains of this type have been tested Tillett and Garner obtained negative results with two strains, and Stewart described anhemolytic strains as being negative in fibrinolytic tests

Double-zoned hemolytic streptococci Brown has described strains having this characteristic appearance when cultivated in blood agar Strains of this type have been derived from both human and animal sources The author has tested some of them, obtained through the courtesy of Dr Brown, and found them to be nonfibrinolytic

Dissociants of streptococci Mellon and Cooper (42) described the action of various dissociants which they obtained from individual strains of hemolytic streptococci Some of the dissociated forms were described as nonhemolytic diphtheroids The variants, which caused only partial lysis in 24 hours, were definitely less active in liquefying fibrin than the original cultures The authors also state that diphtheroids with acid-fast granules considered to be in the tubercle bacillus cycle were indistinguishable in their fibrinolytic activity from diphtheroids dissociated from streptococci Subsequent reference will be made, in relation to virulence, to the findings of Tunnicliff (68), who noted the loss of lytic activity by certain strains associated with the change from cultures producing smooth colonies to those producing rough irregular colonies, and also to the results obtained by Dawson and his coworkers (7), who, with M, S, and R variants of the same strain, observed no difference in fibrinolysis, each of the cultures being active

e Staphylococcus and other bacterial species

The effect of staphylococci on the coagulation of blood and the dissolution of fibrin has received the attention of many investigators It is not within the scope of this review to consider these

properties of staphylococci, because the slowly liquefying action of staphylococci on fibrin, although constituting an example of bacterial fibrinolysis, differs in many respects from the rapid fibrin-dissolving effect of hemolytic streptococci Madison (35) has described the immunological differences between the products of the two bacterial species

Concerning other bacterial species, the available reports are limited Tillett and Garner tested several members of the colon-typhoid group and also *Hemophilus influenzae* and found them to be nonfibrinolytic Schmidt tested a heterogeneous group of organisms and obtained uniformly negative results Madison (40), however, obtained interesting results with *B pestis* Sixteen strains were tested for fibrinolytic activity One of them was of human origin (20 years old), and the others were derived from field mice and ground squirrels For the fibrin clot, he used fibrinogen and thrombin obtained from the plasma of man, guinea pig, rabbit, rat, and other animal species Using methods of titration which he described, Madison found that the cultures of *B pestis* induced lysis of the fibrin-clots from the blood of several of the animal species, including man The potency of lytic action was, however, greatest against the coagulum of rat's blood

Fisher (11a) in studying the fibrinolytic properties of staphylococci noted that certain contaminating bacterial species dissolved plasma-clot slowly in one to six days The strains consisted of *B subtilis* (5 strains), and single cultures of *B proteus*, *B pyocaneus*, diphtheroids, and *B alkaligenes* Owing to the fact that an incubation period of several days was necessary before dissolution occurred, the possibility that the liquefaction might be dependent upon proteolytic digestion warrants consideration No studies dealing with this point have been made

Weiss (80a) made observations with two strains of *Bacterium melaninogenicum* The cultural material was concentrated through alcoholic precipitation, and the tests were made with human fibrinogen-thrombin preparations A 1 to 4 dilution of the concentrate of one strain caused lysis in forty minutes, while original concentrations of the other strain caused partial lysis (designated 2+)

Neter and Witebsky (48) found that *Pneumococcus* behaved like *Streptococcus viridans* in fibrinolytic studies with dextrose-broth cultures Tillett and Garner, Schmidt, Lippard and Johnson, and others could demonstrate no fibrinolysis with pneumococci

III CORRELATION OF FIBRINOLYTIC ACTIVITY WITH OTHER BIOLOGICAL PROPERTIES OF HEMOLYTIC STREPTOCOCCI

a Relation to proteolysis

Laca and Porzecanski studied the proteolytic, fibrinolytic, and hemolytic activity of ninety-six strains of streptococci They found all of these properties commonly present in many of the pathogenic strains However, in certain strains, fibrinolysis was present but proteolysis was absent, while in others the proteolytic effect was marked, but fibrin dissolution did not occur Garner and Tillett by determinations of amino nitrogen contrasted the action on fibrin of fibrinolysin and streptococcal peptase

b Relation to the production of hemolysin and of toxin

With respect to the qualitative differences of hemolysins of streptococci, since the relationship is contained in the reports listed under the kinds of streptococci classified on the basis of their action on blood agar, the results need not be restated Among strains of hemolytic streptococci of the *beta* type, accurate comparative measurements of hemolysin and fibrinolysin have not been made However, on the basis of the size of the zone of hemolysis created by colonies in blood agar, Hadfield and associates, Schmidt, and others have stated that no strict relationship exists between potency of strains in the production of hemolysin and of fibrinolysin

Fraser and Madison using scarlatinal strains attempted to correlate fibrinolytic activity, toxin production, and severity of scarlet fever They found a 63 per cent correlation between the titre of toxin produced by the strains and severity of disease graded according to degree of fever, duration, and complications On the same basis they reported an 80 per cent correlation between the titre of fibrinolysin and severity of illness They stated that their results agreed with the conclusions of Dack and

his associates that a high fibrinolytic titre is significant in relation to the complications of scarlet fever

Morales-Otero and Pomales-Lebron (44) compared fibrinolytic activity with toxigenicity as determined by intracutaneous tests in the shaved skin of white goats Of fifteen strains, thirteen were both toxigenic and fibrinolytic

c Relation to virulence

The types of illnesses resulting from hemolytic streptococcus infections are characteristically diverse The manifestations of the diseases range from clinical entities, the etiology of which may be diagnosed or suspected without laboratory aid, to other disorders which have characteristics common to many pyogenic infections The mechanisms of hemolytic streptococcus infections appear to involve properties which are integral parts of the bacterial cell body, such as capsule formation, and perhaps others, and also substances which are elaborated and excreted by the organisms That hemolytic streptococci produce different kinds of noxious agents is evidenced by many reports and is particularly well illustrated by the erythrogenic toxin and the hemolysin These substances possess different properties and have been studied as separate entities, although elaborated by the same types of organism Furthermore, with the possible exception of the studies of Mudd and his associates (3), the occurrence of the excretory products in strains has not, up to the present time, been found to parallel any individual constituent of the bacterial cell structure Concerning the production of fibrinolysin by streptococci in relation to structural characteristics of the organisms, a few observations have been made Hadfield Magee, and Perry observed with two strains, which produced matt colonies (virulent) at the time of high fibrinolytic activity, that the subsequent change to cultures producing glossy colonies (avirulent) was attended with marked reduction in the production of fibrinolysin They found the average virulence for mice of their strains most potent in the production of fibrinolysin was higher than that of the least active Tunnicliff (71) reported that strongest lytic action was associated with virulent strains

which possessed capsules and produced smooth colonies She found that the production of fibrinolysin was lost when cultures were altered by dissociation so that granular colonies with irregular edges were formed She reported further that reversion of strains to the type which formed smooth colonies, was accompanied by the restoration of active fibrinolysis Schmidt noted the loss of lytic activity with some strains after repeated subculture, and that virulence for mice was also lost When, however, by mouse passage, virulence was restored, lytic action also increased

Morales-Otero and Pomales-Lebron (44) cited their experience with strains which were virulent for mice at a time when the organisms were fibrinolytic Two years later, the same strains had lost mouse virulence but had retained fibrinolytic and toxigenic powers Dawson, Hobby, and Olmstead in describing the results of their extensive studies on M, S, and R variants of hemolytic streptococci briefly record, without giving details, that no significant differences were observed in the fibrinolytic capacity of the three variants of the same strain

These findings indicate that although the production of fibrinolysin by hemolytic streptococci may frequently accompany the presence of experimental indices of pathogenicity (colonial structure and virulence for mice), the relationship is not an inseparable one In the author's experience, strains of hemolytic streptococci of highest fibrinolytic potency may not be virulent for mice Furthermore, as will be subsequently discussed, human strains of hemolytic streptococci are not regularly capable of causing dissolution of the fibrin of mouse's blood Since the presumptive evidence of the rôle of fibrinolysin in virulence is derived from the capacity of the invading organism to dissolve the fibrin of the infected animal, it follows that invasion in the absence of fibrin susceptibility is referable to other conditions In experimental infections, the mechanism of virulence often centers around factors which involve susceptibility or resistance to phagocytosis These same factors, in all probability, play an important and often decisive rôle in human infections However, in infections in man due to hemolytic streptococci supple-

mentary factors may influence the pathogenesis of the diseases For example, the erythrogenic toxin, which seems to be of limited significance in infections of laboratory animals, induces toxic manifestations in man Whether or not the fibrinolytic properties of human pathogenic strains may also be a contributing factor to some of the characteristic elements of hemolytic streptococcus infections, has not been determined but may be surmised from suggestive indirect evidence

Neter (50) found the fibrinolysin present in the spinal fluids of four out of five patients with meningitis due to hemolytic streptococci With samples of the spinal fluids of cases of meningitis due to other organisms, he obtained negative results, except in one case of pneumococcus meningitis He also reported the occurrence of lytic activity in peritoneal and pleural exudates from hemolytic streptococcus infections, and with the pericardial and peritoneal fluids from *Staphylococcus aureus* infections He examined also the peritoneal exudate of mice infected with hemolytic streptococci and pneumococci In the infections with streptococci, the peritoneal washings induced lysis of human fibrin, but the material from mice infected with pneumococci was negative

In connection with the production *in vivo* of fibrinolysin, it may also be mentioned that the thinness of the fluid so characteristic of the exudate obtained early in cases of infections of the serous cavities due to hemolytic streptococci, particularly empyema, appears to be due to the lytic action of the infecting organisms on the fibrinous exudate Goodpasture in describing the pathological changes occurring in bronchopneumonia due to hemolytic streptococci of 1917–18 refers to cases in which "microscopically the alveoli are filled with polymorphonuclear leukocytes and usually enormous numbers of streptococci, with little or no fibrin" In MacCallum's account of pneumonia during the World War, reference was not infrequently made to areas in which fibrin was scarce or absent The density of fibrin deposits in many of the lesions was also commented upon The present writer has examined material from two cases of empyema Fibrinolysin was demonstrable in the thin pleural fluid obtained early in the disease However, as the exudate became thick with

fibrin, antifibrinolytic properties were demonstrable in the blood of the patients It seems not unlikely that the pathogenesis of some aspects of hemolytic streptococcus infections may be explained on the basis of the fibrinolytic potency of the organism in relation to the antifibrinolytic properties of the host

IV HEMOLYTIC STREPTOCOCCI FROM ANIMAL SOURCES, WITH PARTICULAR REFERENCE TO ACTION ON FIBRIN OF BLOOD FROM DIFFERENT ANIMAL SPECIES

Tillett and Garner reported that, although cultures of hemolytic streptococci derived from patients caused lysis of normal human fibrin-clot, normal rabbit fibrin-clot was resistant to dissolution when tested under comparable conditions Observations concerning differences in fibrinolytic activity referable to animal sources of fibrin have yielded interesting results

Van Deventer and Reich (73) tested three human strains and two animal strains (P 454 and K 158 E of Lancefield) against the plasma-clot of the following animals rabbit, guinea pig, rat, domestic fowl, horse, cow, goat, sheep, dog, and cat All tests were negative The three human strains were lytic for human fibrin They were also tested against the plasma-clot of rhesus monkeys Two of the strains caused lysis of monkey fibrin but at a slower rate than the effect on human fibrin One of them was equally active against human and monkey fibrin

Madison (34) tested twelve strains of hemolytic streptococci derived from horses suffering from strangles against samples of fibrin derived from horse, man, hog, cow, and rabbit Fibrinogen-thrombin preparations were employed because of their greater susceptibility to lytic action The equine strains caused dissolution of horse fibrin but did not liquefy the fibrins derived from the other animal species, including man Two human strains of hemolytic streptococci were weakly lytic against *horse* fibrin In addition, Madison found that three strains of hemolytic streptococci obtained from hogs (septicemia) were highly active against *hog* fibrin The same strains were weakly active against *human* fibrin, but negative against the fibrin of the other animal species

Planet also compared the action of *human* and *equine* strains of

hemolytic streptococci against the plasma-fibrin of *human* and *equine* sources The single *human* strain of hemolytic streptococcus, which he employed, caused dissolution of the fibrin from five different *human* plasmas, but was inactive against the fibrin of twenty-two different *horse* plasmas One of his *equine* strains caused lysis of all of the samples of fibrin from *horses* but was negative against *human* fibrin-clot With other *equine* strains of hemolytic streptococci, varying degrees of lytic activity for *equine* fibrin were noted but no alteration of human fibrin occurred Some of the equine strains fermented lactose and some did not No relationship was noted between the fermenting activity and fibrinolytic capacity

Smith, Hankinson, and Mudge tested twenty-two strains of hemolytic streptococci derived from *cow's milk* against the plasma-clot of *bovine* blood Nine of the strains caused varying degrees of lysis of *bovine* fibrin Of these, two were from normal cows, five were from cows with mastitis in a quarter other than that which supplied the infected milk, and two were from cows with chronic mastitis The results were not conclusive, but suggested the possibility that strains lytic for bovine fibrin might be significant in mastitis

Pilot, Buck, and Davis (53) examined one hundred strains of hemolytic streptococci obtained from the tonsils of cows, and ninety-two gave negative fibrinolytic tests with human fibrin Among forty-three strains derived from the tonsils of hogs, thirty-nine were negative No report was made of tests made with fibrin of the cow or hog In a subsequent article twenty-two canine strains were reported as negative for human fibrin (54)

Seegal, Heller, and Jablonowitz in a study of hemolytic streptococci derived from *monkeys*, tested the fibrinolytic activity of the cultures against fibrin from *man* and from *monkeys* Nineteen Group A strains caused lysis of *human* fibrin within $3\frac{1}{2}$ hours, and also dissolved *monkey* fibrin but at a slower rate, ranging from 6 hours with 3 strains to a negative result with 2 others With four Group C and five Group G strains, *human* fibrin was liquefied regularly within $3\frac{1}{4}$ hours, and lysis of *monkey* fibrin occurred with the same prolonged rate of activity obtained with

the Group A strains The lysis of *human* fibrin was uniformly more efficient than that of *monkey* fibrin

Yen, in studying the problem of the resistance of animal fibrins to dissolution, observed the influence of quantitative factors in the reaction Using hemolytic streptococci from patients, he concentrated the fibrinolysin from filtrates of cultures by alcoholic precipitation In order to have a more sensitive substrate, he employed fibrinogen-thrombin preparations isolated from the plasma of man, rabbit, and guinea pig He found that human fibrin was dissolved in 3 to 5 minutes, that rabbit fibrin was liquefied in 30 to 180 minutes, and that guinea pig fibrin failed to liquefy He concluded that rabbit fibrin-clot was not absolutely resistant to lysis by human strains of hemolytic streptococci, if sufficiently high concentrations of fibrinolysin were tested

Schmidt also emphasized the importance of the quantitative factor in determining the results obtained with materials from different animal species Although he found exceptions with some strains, he confirmed the findings of others with respect to the homologous source of materials, *provided* the usual test dose of culture was used and the fibrin was contained in the clot of whole plasma When, however, large amounts of fibrinolysin were employed and added to fibrinogen-thrombin preparations, the principle of species specificity was not regularly maintained

Concerning the sensitivity of the fibrin substrate to dissolution by streptococci, an additional complicating factor is introduced when the fibrinogen constituent of the clot and the thrombin component are each derived from a different animal species Tillett and Garner reported that when fibrinogen from rabbit's blood was coagulated in the presence of cultures of hemolytic streptococci by thrombin from human blood, dissolution occurred, also, when fibrinogen of human blood was clotted by thrombin of rabbit's blood, liquefaction took place When, however, both constituents of the coagulum were derived from the rabbit, the results were either negative or slow dissolution occurred after many hours In the above experiments, the determining factor in the occurrence of active fibrinolysis was the

presence of at least one human element in the fibrinogen-thrombin complex

Madison (36) used materials which he designated as "hybrid fibrins" He derived fibrinogen and thrombin from eleven different animal species, including man Using a human strain of hemolytic streptococcus, he found that dissolution occurred in every instance when the fibrinogen component of the fibrin was of human derivation regardless of the source of the thrombin When the human component was thrombin and the fibrinogens were from various animals, dissolution occurred, but proceeded at a slower rate than the control of human fibrin If neither constituent was of human origin, the results were negative Comparable but somewhat less striking homologous species relationships were found to exist when an equine strain active against horse fibrin, and a porcine strain active against hog fibrin, were tested with hybrid fibrins In these latter experiments, however, there were some irregularities not explicable on the basis of the individual animal source of the materials

The subject of hybrid fibrins is obviously a somewhat confused one Schmidt emphasized the importance of the quantitative proportions between fibrinolysin and fibrin substrate He found that small doses of a highly active human strain acted only upon fibrins when one element was of human origin When the amount of fibrinolysin was increased, however, some of the hybrid fibrins were dissolved Schmidt extended the studies by considering whether or not strains, which are highly pathogenic for a given species, would dissolve the fibrin of the species provided thrombin of the homologous animal was employed His results were not harmonious They conformed to a homologous species relationship between virulence and source of fibrin with some strains, but the correlation was not demonstrable with others For example, he described an equine strain, virulent for mice, which liquefied mouse fibrin formed with horse thrombin, but was inactive against mouse fibrin formed with human thrombin

It is obviously impossible in the present state of knowledge to interpret clearly the results obtained with the manifold hybrid fibrins It seems probable that the results are dependent upon

quantitative factors in some instances, and upon qualitative differences of materials in others However, even when the differences are quantitative, homologous fibrin has been found to be more susceptible than heterologous material Viewed as a chemical reaction involving a system consisting of enzyme (fibrinolysin) and substrate (fibrin), variations in sensitivity are dependent upon the sources of materials, but the degree of specificity necessary to elicit the dissolving effect is not established Furthermore, since the materials used are not chemically pure, accessory factors, which may influence enzyme systems such as the fibrinolytic process, merit consideration Additional information on this complex subject seems to require chemical procedures which are more technically exact than the methods employed at present

In spite of the limitations on the interpretation of the results just discussed, the apparent predilection of strains of hemolytic streptococci for the fibrin of a species homologous to that in which the organisms may survive, and in some instances invade, contains implications of biological interest which invite additional study

V CHARACTERIZATION OF FIBRINOLYSIN AND NATURE OF THE REACTION

The fibrinolysin has been found to be freely excreted by the living, growing bacterial cells Consequently, it has been possible to obtain active fibrinolytic material, free from the microorganisms, by filtration Garner and Tillett found that the fibrinolytic principle could be partially purified by (a) precipitation of culture filtrate with 3 volumes of 95 per cent alcohol, and, (b) adsorption on polyaluminum hydroxide B of Willstätter followed by elution with M/10 sodium phosphate buffer, pH 7 3 Concentration was accomplished by dissolution of the precipitates in small quantities of solvent, but was best obtained by vacuum dialysis (15) Concentration by alcoholic precipitation has also been reported by Madison (33), Yen, and Schmidt

It should be noted that when high degrees of concentration are attempted, preparations may be encountered, which inhibit

coagulation The explanation of the anticoagulative effect is not clear It seems possible that it may be referable to some other constituent of the filtrate which is also concentrated together with the fibrinolysin For example, peptone is known to contain anticoagulating material, which might be responsible for the effect It seems also possible that inhibition of the clotting process might be dependent upon the physico-chemical action of highly concentrated proteins or other organic materials

Garner and Tillett found that active culture filtrates were relatively heat stable, in some instances resisting heat of 100°C for 60 minutes Dennis and Berberian (9) reported that fibrinolytic activity was markedly weakened by boiling for one-half hour In contrast to the heat stability of culture filtrates, Garner and Tillett observed the activity of material obtained by alcoholic precipitation was destroyed at 57°C for one hour However, when the fibrinolytic agent was purified by adsorption and elution, the resultant material was again heat-stable as in the case of the culture filtrate The sensitivity of the material obtained by alcoholic precipitation suggests that the procedure separated the active principle from other substances which afforded protection from the deleterious effects of heat Although an explanation of the differences in the effect of heat is not clear, the thermal properties suggest that the fibrinolysins of different preparations may exhibit variations in sensitivity to other inactivating substances, such as chemicals or antisera

The fibrinolysin conforms in many of its characteristics to a protein The partially purified materials give positive tests for protein, and fibrinolytic activity is destroyed by digestion with trypsin or papain (15)

Using fibrinogen-thrombin preparations, Garner and Tillett found that the fibrinolysin was not bound to the reaction products, since the active material was recovered approximately quantitatively after dissolution of fibrin was complete

In characterizing the fibrinolysin, therefore, on the basis of the data available at present, the active agent may be considered to be enzymic in nature for the following reasons 1 It is of biological origin 2 Catalytic property is indicated by the fact

that active material is recoverable, approximately quantitatively, after the reaction is completed 3 Destruction by heat (high temperatures for broth filtrate, low temperature for material isolated by alcoholic precipitation) 4 Tests for protein are positive

The fibrinolysin differs, however, from proteolytic enzymes in that preparations of the former exert no hydrolytic action on casein, gelatin, or peptone Furthermore, it also differs from the so-called streptococcal peptase, which is obtained by rupturing the bacterial cells and which acts upon casein but is especially vigorous against peptone (15)

Fibrinogen is the only substrate besides fibrin which has so far been found to be susceptible to fibrinolysin Demonstration of the action on *human* fibrinogen was made in experiments (15) in which fibrinogen, incubated for short periods with fibrinolytic cultures, was incapable of forming fibrin following the subsequent addition of thrombin *Rabbit* fibrinogen, however, in parallel experiments, retained the capacity to form fibrin even after preliminary incubation of eighteen hours with fibrinolysin

One of the interesting features of the fibrinolytic phenomenon concerns the nature of the end products of the reaction Following dissolution of fibrin and during subsequent incubation, determinations have been made of increases in amino N (Garner and Tillett), and also of non-protein N and of the evolution of ammonia (Garner) It was found that, during the experimental period, there is a small and gradual increase in the amino N content of the solution The results contrast, however, in degree very markedly with the observed effect of trypsin on fibrinogen, where the sharp increase in amino N, characteristic of proteolytic fermentation, occurred Whether or not the action of fibrinolysin is accompanied by proteolytic hydrolysis, is not clear The end products appear to be protein but to have somewhat different properties from fibrinogen with respect to thermal precipitation point and the precipitating concentration of salts Garner did not detect the evolution of ammonia during the experimental period

From these experiments it seems likely that the chemical deg-

radation of the highly complex molecules of fibrin is not great, even though the physical change of solid fibrin into a solution is striking

In referring to the observations of Garner and Tillett, Jablonowitz calls attention to the fact that globulin present in the impure preparations of fibrinogen may have accounted for the properties of the end products of the reaction rather than a change in the characteristics of fibrinogen to globulin through the action of fibrinolysin Jablonowitz studied the alterations in the immunological specificity of fibrinogen following the action of fibrinolysin derived from a strain of hemolytic streptococcus of human origin For purposes of obtaining highly purified material, he prepared fibrinogen by methods of repeated precipitation This material, when tested against the antiglobulin serum described by Kendall, gave only a very faint reaction Consequently it was used in the immunization of rabbits The sera of the immunized rabbits was tested against two preparations (a) sterile broth + fibrinogen, (b) fibrinolysin + fibrinogen The two mixtures (a and b) were incubated for 24 hours at 37°C before being used in precipitation tests with antifibrinogen serum After the precipitation tests had been incubated, the precipitates were centrifuged, washed, and analyzed for total N The total N in the precipitate produced with fibrinolysin + fibrinogen (b) was less (0 075 mgm) than that obtained from the sterile broth + fibrinogen mixture (0 31 mgm) Jablonowitz concluded therefore that fibrinogen was altered immunologically by the action of fibrinolysin In other experiments to determine the rate of alteration, he found that there was an initial lag period of approximately 15 minutes followed by a rapid change which seemed to be complete in about an hour

Garner (16) reported that the end product was not differentiated from fibrinogen by serological reactions The findings, on which that observation was based, were obtained by Garner and Tillett (unpublished) in determining the precipitative titre by the usual technique Using progressive dilutions of precipitinogen, the differences in the end points of the tests with fibrinogen and dissolved fibrin were not sufficiently great to indicate differences in the precipitinogenic preparations

Doudoroff investigated the effect on fibrinolytic filtrate of cultures of various bacterial species After mixing 48 hour cultures with the filtrate, he subsequently killed with chloroform the organisms which had been added and tested the mixture for fibrinolytic action He found that the fibrinolysin was most regularly inactivated by bacteria which were capable of liquefying gelatin The inactivating effect of the cultures was usually destroyed by heating at 60°C for 30 minutes

Madison and Snow (41) tested the antifibrinolytic effect of several antiseptics which they employed in sub-bacteriostatic doses in cultures The results were not striking They also added antiseptics to fibrinolytic tests and concluded that tincture of iodine impaired lytic action more definitely than other drugs Huntington cultivated strains of hemolytic streptococci in 0 05 per cent glucose-broth with and without 20 mg per cent of sulfanilamide, and was unable to observe any deleterious effect upon the production of fibrinolysin by the drug

VI IMMUNOLOGICAL STUDIES

In immunological studies, oxalated plasma from the blood of normal individuals and patients has been most regularly employed By this procedure, the measure of antifibrinolytic resistance is made with the fibrin of the patient's blood in the presence of whatever antifibrinolytic properties may be concomitantly contained in the additional constituents of the same sample of plasma Serum has also been employed as in other immunological reactions However, owing to special conditions of the tests, which will be referred to later, the serological method has not been regularly adopted

Although 0 2 cc of plasma has been usually employed, inquiry has been made into the possible significance of differences in the amount of fibrinogen contained in blood in different diseases Hadfield and associates investigated this point and found that the content of fibrin in plasma did not appreciably affect the dissolution time, even when as much as 1400 mgm per 100 cc of blood was present Van Deventer (75) concentrated fibrinogen fourfold and found that the speed of dissolution was slowed but did not result in complete refractoriness From these find-

ings, it seems unlikely that, under the condition of usual tests, significant variations in the dissolution time are referable to the quantities of fibrin in the blood

The value of using strains of hemolytic streptococci of highly potent fibrinolytic activity in antifibrinolytic tests has been advocated by investigators of the subject In order to emphasize the difference between the results obtained with normal susceptible fibrin and patients' resistant plasma-clot, Tillett, Edwards, and Garner (66) employed the whole broth culture of a strain of maximum potency By this procedure the greatest amount of fibrinolysin was contained in the test material, including such additional amounts as the living organisms might produce during the period of incubation

Hadfield and his co-workers considered the use of a powerfully lytic strain important in differentiating between the rate of dissolution of normal fibrin and of that from patients Stuart-Harris (63), using data derived from titration experiments, illustrated graphically the characteristic curve of the relationship between concentration of lytic agent and time required for fibrinolysis On the basis of the ratios obtained, he concluded that the use of weakly active strains or high dilutions of potent strains so prolonged the dissolution time with normal fibrin that the assay of the degree of resistance in patients' fibrin was masked Furthermore, differences between samples of fibrin, which were minor when potent material was used, were unduly emphasized when weakly acting preparations were employed Other observers have employed three to five strains in each test and used the average results

Limited consideration has been given to the possibility that the fibrinolysins of different disease-producing strains may be immunologically distinct Tillett, Edwards, and Garner tested the blood of a few patients with the homologous strain derived from each patient but were unable to detect any difference in antifibrinolytic resistance Van Deventer (74) tested forty strains against the fibrin of three normal persons and two resistant patients He concluded that there was only one type of fibrinolysin among the strains Yu and Zia described their findings with

plasma from a patient convalescent from scarlet fever which was shown to be resistant to a strain from a case of puerperal sepsis, but susceptible when tested with some of the scarlet fever strains They did not clearly indicate whether all of the test strains possessed the same degree of fibrinolytic potency At the present time, among human strains no definite evidence of immunological differences of the fibrinolysins has been obtained, although an exhaustive study of the subject has not been made

Determinations of the presence or absence of resistance have been made by contrasting the brief length of time required to liquefy normal fibrin with either the absence of any dissolving effect on patients' fibrin or the prolonged period necessary to effect liquefaction The three variables in fibrinolytic tests are quantity of fibrin, quantity of fibrinolysin, time required for dissolution Fibrinolytic "units" have been suggested by some observers Madison and Taranik (39) proposed that the highest serial dilution of broth culture causing complete liquefaction of the fibrinogen-thrombin clot by the end of two hours incubation be assumed to contain one fibrinolytic unit From the dilution, the number of lytic units per cubic centimeter of broth culture was calculated Van Deventer (76) referred to a unit of fibrinolysin as three times the amount necessary to dissolve, within two hours, the fibrin of fibrinogen-thrombin preparations Standards, however, have not been used extensively enough in studies of antifibrinolysin to be evaluated A sufficient amount of information is not yet available concerning methods of quantitative measurement and the mechanism of the reaction to make improved procedures practicable Consequently, estimations of resistance based on the factor of time has been most widely used From the standpoint of exact quantitative measurements, the limit of experimental error is in all probability relatively broad For this reason, rates of dissolution which might serve as sharp dividing line between normal and abnormal results have not been advocated In the absence of arbitrary standards, most observers have, with minor variations, employed the following scheme for estimating degrees of resistance, when the amount of culture and plasma were kept constant Dissolution

in less than one hour indicates susceptibility, dissolution in one to three hours indicates doubtful to weak resistance, dissolution requiring three hours or longer up to twenty-four hours indicates "definite" or "marked" or "partial" resistance, no dissolution during the twenty-four hour period of the test indicates "maximum" resistance When several tests are set up with constant quantities of the same samples of plasma and culture, the dissolution time is constant within a narrow range of variation Consequently, when the difference in time of liquefaction of two separate specimens of blood is a matter of several hours, the delayed rate assumes significance

Concerning the susceptibility of the fibrin from normal persons, a sufficient amount of information has accumulated to indicate the average findings among healthy adults Among thirty normal individuals, Tillett, Edwards, and Garner (66) found the dissolution time to be 8 to 15 minutes in thirteen instances, 15 to 60 minutes in eight tests, and from one to four hours with nine specimens Morales-Otero and Pomales-Lebron (46) found that the time required for dissolution varied in tests with normal fibrin from 30 minutes to two and a half hours Myers, Keefer, and Holmes reported that the average time for lysis with samples of blood from fourteen adults was one hour, the minimum time being 14 minutes and the maximum five hours Waaler (78) stated that of tests made with specimens of blood from thirty-nine normal persons, thirty-four were classed as susceptible, and five as partially resistant In a second article by Waaler (79) the blood of fifty of fifty-five normals were found to be susceptible and five partially resistant Hadfield and associates stated that in tests with specimens of blood from twenty-eight adults none was totally resistant Stuart-Harris (64) found the fibrin from 98 6 per cent of seventy-two persons to be susceptible From his average results, and using a factor of standard deviation, he placed the limit of time for normal tests at 51 minutes

From these results it may be seen that lysis of the fibrin clot of the blood of the great majority of normal individuals occurs in less than one hour, and commonly requires a considerably shorter time Although each of the investigators has employed individ-

ual strains selected for the purpose but not standardized on the basis of any arbitrarily adopted unit of accurate measurement of of fibrinolytic potency, the results are in general agreement On the basis of these findings, it may be estimated that the blood of approximately 85 to 90 per cent of normal healthy individuals may be arbitrarily classified as susceptible on the basis of tests in which the dissolution time is less than one hour In tests made with the blood of normal children, the data for age groups ranging from three to fifteen years of age are consonant with the findings in adults Owing, however, to the frequency of upper respiratory infections in children during the winter months, it has been suggested that varying degrees of resistance may occur more frequently than in adults Among the acute diseases, directly referable to infection with hemolytic streptococci, immunological studies of the following conditions have been reported Acute tonsillitis, with and without extension to mastoid, middle ear, or sinuses, scarlet fever, with and without complications, erysipelas, suppurative infections such as empyema, peritonitis, and abscesses in different locations, septicemia arising from different sources The data to be given were obtained by consolidating all of the findings presented by various authors Although the averages are not entirely in accord with the individual findings of each report, the differences are not sufficiently great to warrant a separate account of each

In immunological studies it has been found that the development of antifibrinolytic properties may be demonstrable at variable times during the course of the disease up to as late as the third or fourth week in convalescence The summarizing data which follow are in many instances derived from repeated examinations of the blood during acute illness and convalescence However, in some of the cases, only one or two tests were made The conclusions, therefore, are to some extent based on partially complete results which limit final conclusions

Acute tonsillitis Tillett, Edwards, and Garner (66), Myers Keefer, and Holmes, Stuart-Harris (63, 64), and Tillett (68) have reported results obtained in forty-eight cases In thirty-two of

the patients (67 per cent) an antifibrinolytic response was noted during convalescence The time in the course of the disease at which the specific resistance developed varied from the first week to as late as the fifth week In uncomplicated cases, the period of lag between the cessation of active disease and the detection of antifibrinolytic properties in the blood usually ranged from two to four weeks

The degree of antifibrinolytic response was also found to vary in individual cases The severity and extent of the infection were not infrequently found to be important factors not only in evoking the development of high antifibrinolytic response, but also in shortening the time of appearance of the specific immunity

Scarlet fever Tillett and associates, in eight cases, Dack and associates in forty-seven cases, Stuart-Harris, in fifty-eight cases, and Waaler in fifty-seven cases found the blood of 86 (50 per cent) out of 170 cases to possess antifibrinolytic properties, observed in most instances during convalescence As in the patients with acute tonsillitis, the results in scarlet fever indicated that the development of antifibrinolysis becomes demonstrable usually within two to five weeks after the cessation of active disease Dack and his associates noted that in the first test with some of the patients, the dissolution time was prolonged Waaler found antifibrinolytic properties more frequently in cases complicated by otitis media and nephritis than in cases with adenitis or arthritis Stuart-Harris obtained antifibrinolysis most frequently in cases complicated by arthritis, carditis, and nephritis

In view of the fact that scarlet fever in recent years has been relatively mild, it seems reasonable to presume that the somewhat less frequent occurrence of antifibrinolytic immunity in patients with scarlet fever (50 per cent) than in those with acute tonsillitis (67 per cent) may be ascribed to differences in the severity of the infections Cases of scarlet fever are usually hospitalized regardless of the degree of illness, whereas only relatively severe cases of acute follicular tonsillitis seek admission to hospitals, and become available for study The reports,

referred to earlier, that scarlatinal strains of hemolytic streptococci possess less fibrinolytic potency than other strains, also suggests limitations in the antigenicity of the fibrinolysin

Erysipelas Combining the results obtained in different laboratories (66, 47, 63), resistance to fibrinolysis developed in 37 (80 per cent) of forty-six patients Tillett, Edwards, and Garner noted that the development of resistance coincided in some cases with the cessation of the spread of the lesion However, in other instances, the same authors observed a delay of one to three weeks in demonstrable antifibrinolysis In ten of their cases, Myers, Keefer, and Holmes noted a high degree of resistance which was present during the period of active disease and persisted after recovery In general, the antifibrinolytic response appeared in erysipelas at an earlier time during the course of the disease than in the uncomplicated cases of either acute tonsillitis or scarlet fever

Suppurative infections with and without septicemia This group includes cases of unusual severity In some of the patients the occurrence of septicemia was reported The mortality rate was high In six fatal cases with septicemia, Tillett (68) found that none developed antifibrinolytic immunity The patients died between the 6th and 25th day of disease It is apparent that the survival period may not have been long enough to permit the appearance of the immune response However, the limited data suggest that the occurrence of antifibrinolysis is less frequent in overwhelming infections than in local processes Dack and his associates reported among the patients with scarlet fever, one fatal case in which the blood contained a high degree of antifibrinolytic resistance Myers, Keefer, and Holmes also described a case which ended fatally with maximum resistance to lysis present in the blood Of six cases with septicemia, which recovered, (4 reported by Tillett, and 2 by Stuart-Harris), three developed antifibrinolytic properties, the fibrin-clots of the other three remained susceptible even after the infection was overcome

The findings with a miscellaneous group of infections, including cellulitis, empyema, mastoiditis, peritonsillar abscess, etc may be collected from the several articles dealing with anti-

fibrinolytic immunity Of twenty-two such cases, sixteen (73 per cent) developed the specific immune response

A summary of the results just given is as follows

	Number of cases	*Resistance present in per cent*
Acute tonsillitis	48	67
Scarlet fever	170	50
Erysipelas	46	80
Miscellaneous	22	73
Septicemia with recovery	6	50
Fatal cases	8	25
Normal individuals	165*	10–15

* Approximate

In a large number of the observations just summarized, the changes in the reaction of fibrin from susceptibility to resistance were demonstrated during the course of the diseases The findings in serial tests with samples of blood from patients, who recovered, indicate that the fibrinolytic substance is frequently antigenic under the conditions of naturally occurring infections, and that the antifibrinolytic response is a specific immune reaction However, additional observations suggest that insusceptibility to lysis may occur under conditions which are not referable to specific antibody response The interpretation of single tests, carried out with plasma obtained during phases of active disease and convalescence will be subsequently discussed

Furcolow and Fousek (14) performed eighty-four tests on seventy patients In twenty-two instances no antifibrinolytic resistance was present None of the latter had proven hemolytic streptococcus disease, although six were suspected In twenty-five tests, the dissolution time ranged from one to three hours, which was interpreted as a suggestive but doubtful indication of resistance Twenty-two of the patients either had proven hemolytic streptococcus infections or had been contacts Antifibrinolytic resistance was marked in thirty-seven tests Thirty-six of the cases had proven hemolytic streptococcus infections

Rheumatic fever Hadfield, Magee, and Perry made tests with the blood of forty-four children with rheumatic disease The patients were divided into a group of twenty-one who had had recent active disease, and a second group of twenty-three quiescent cases The first group was further subdivided into nine cases with sedimentation rate (red blood cells) above 20 Among them, five exhibited either maximum or partial resistance Of twelve cases having had recent attacks but with a sedimentation rate below 20, five had maximum or partial resistance The blood from each of the quiescent cases was susceptible to lysis

Myers, Keefer, and Holmes made observations on thirty-four cases of rheumatic fever, twenty-nine of which either gave a history of a recent attack of acute respiratory infection or carried hemolytic streptococci in their throats at the time of admission to the hospital Of these twenty-nine cases, the blood in twenty-seven possessed maximal antifibrinolytic resistance Of the five remaining patients who had active disease but who gave no evidence either by history or by throat culture of having had hemolytic streptococcus infection, four possessed either maximal or partial resistance The average time required for lysis in all the tests was nineteen hours

Stuart-Harris (64) among twenty-two convalescent cases, found partial resistance in seven (32 per cent), and in tests with the blood of forty-eight active cases, twenty-nine (60 per cent) partial or complete resistance was present

Waaler (79) tested the blood of seven patients with rheumatic fever, all of whom had acute infections of the throat Six possessed maximal or partial resistance He stated that when manifestations of active disease subsided, the antifibrinolytic property of the blood decreased

Tillett (68) examined the blood of eight patients with active rheumatic fever, all of whom had had preceding acute upper respiratory infections Six possessed maximum resistance, and two partial resistance

Lippard and Johnson made observations on the blood of five cases (8 to 15 years of age) The dissolution time varied from 3 hours to maximum resistance The authors also found high

titre of streptolysin antibodies in the same specimens of blood However, the parallelism of antistreptolysin and antifibrinolysin was not quantitative, since the specimens with the highest titre of antistreptolysin did not exhibit the greatest degree of antifibrinolytic resistance Stuart-Harris (63) also brought out the fact that titre of antistreptolysin and antifibrinolysin were not concomitantly present to the same degree

Summary of antifibrinolytic tests in patients with rheumatic fever

	Number of cases	*Resistance present in per cent*
Active disease	123	72
Quiescent disease	45	15

From these results it is interesting to note that the findings obtained with cases of active rheumatic fever demonstrate that the frequency of the development of antifibrinolytic resistance (72 per cent) is essentially the same as that obtained in cases of acute tonsillitis without the visceral manifestations of rheumatic disease (67 per cent) Whether or not the rheumatic process has in itself the capacity to evoke an antifibrinolytic response or or whether upper respiratory tract infections due to hemolytic streptococci occurring frequently in rheumatic subjects elicit resistance to fibrin dissolution cannot be assayed from the results so far available It is not within the scope of this article to discuss the broader subject of the possible relationship of hemolytic streptococci to rheumatic fever However, the observations in cases of acute upper respiratory diseases of hemolytic streptococcal origin and also in cases of active rheumatic fever appear to be sufficiently consonant to indicate that the frequency with which antifibrinolytic properties develop in these disorders is comparable

Rheumatoid arthritis Myers, Keefer, and Holmes tested the blood from eleven cases, two had maximal resistance, and another, following acute sinusitis developed maximal resistance The average dissolution time for the group was six hours, which is somewhat higher than the average of one hour for the normal controls, but considerably less than the average of nineteen hours for the cases of rheumatic fever

Stuart-Harris in sixty cases of rheumatoid arthritis found resistance in six. Among ten cases of other types of chronic arthritis, no resistance was noted

Waaler observed nineteen cases and recorded two as having 3+ resistance, four with 2+ resistance, four graded as 1+, nine were susceptible He considered many of the reactions to be weak but suggested that resistance might have been more frequently encountered if the tests had been performed with samples of blood obtained earlier in the course of the disease Neither the history of respiratory infections nor the results of bacteriological studies were reported

Gonoccocal arthritis Of six cases studied by Myers and associates two had maximum resistance The average dissolution time was five hours which is approximately the same as that obtained in cases of rheumatoid arthritis Tillett and associates found in one case that normal suseptibility remained unchanged in tests repeatedly performed during sixty days of activity and convalescence Stuart-Harris also noted susceptibility in one case of gonoccocal arthritis

Still's disease In five children, Waaler observed no antifibrinolytic resistance

Summary of antifibrinolytic tests in patients with arthritis

	Number of cases	Resistance present in per cent
Rheumatoid arthritis	90	14
Gonococcal arthritis	8	25
Still's disease	5	0

The findings with the arthritic group are significant when contrasted with the results obtained in rheumatic fever It is also interesting to note that the frequency of antifibrinolytic resistance was slightly greater than in normal individuals A discussion of the possible significance of these results will be reserved until the findings in other diseases are described However, from the standpoint of critical analysis, it would appear to be necessary to exclude the possibility of a relatively recent hemolytic streptococcus infection—whether causal or incidental —as the incitant of the antifibrinolytic response in order to

interpret the findings obtained in chronic disorders of uncertain etiology

Acute Nephritis In five patients, all of whom had previously suffered from acute tonsillitis due to hemolytic streptococci, four developed moderate to maximal resistance (68) Waaler (78) commented upon the frequency of antifibrinolysis in seven cases In four cases, Myers and associates found the average dissolution time to be six hours, but did not comment upon the occurrence of antecedent respiratory infection Similarly, Stuart-Harris found the fibrin-clot in two cases to be susceptible No bacteriological details were given

Recurrent tropical lymphangitis Morales-Otero and Pomales-Lebron (46) in a study of the relationship of hemolytic streptococci to tropical lymphangitis tested for the presence of antifibrinolytic properties in the blood of fourteen patients suffering from this disease They found maximum antifibrinolytic resistance in 7 instances, moderate resistance in 3 In the remaining 4 cases resistance was either absent or doubtful They reported that the resistance was usually most marked early in the disease, gradually decreased during convalescence, and rapidly reappeared with a recurring attack of lymphangitis

Bacterial endocarditis Myers and associates studied two cases due to *Streptococcus viridans* from each of which the fibrin-clot exhibited maximum resistance A third case, with infection due to an indifferent streptococcus, was found in repeated examinations to be without antifibrinolytic properties

Waaler (79) tested the blood of four cases which were due to *Streptococcus viridans* Three of the four possessed antifibrinolytic properties A fifth case, due to a fecal streptococcus, gave tests rated as 2+ resistance

Stuart-Harris reported observations in two cases which were due to *Streptococcus viridans* One of the patients, who had previously suffered from rheumatic fever, developed partial resistance The fibrin-clot of the other was susceptible, and at autopsy no signs of rheumatic fever were noted In a third case of undetermined bacterial etiology, no antifibrinolytic resistance was present

The high incidence of antifibrinolytic properties in the blood

of patients with endocarditis due to *Streptococcus viridans* is an interesting finding, the interpretation of which is not apparent If the resistance to lysis is dependent upon the presence of specific immune properties, the antifibrinolytic response appears to be evoked either by green streptococci or in association with the underlying rheumatic disease The possible influence of non-specific factors in antifibrinolysis will be presently considered It is interesting to note in passing that both McEwen and Coburn have reported in personal communications that the antistreptolysin titre of the serum of patients with bacterial endocarditis is usually not increased

Diseases not associated with hemolytic streptococci—It is unnecessary to consider individually the large number of diseases which have been used for comparison with infections due to hemolytic streptococci The control groups have consisted of diseases of diverse bacterial etiology, such as pneumonia, tuberculosis, typhoid fever, diphtheria, staphylococcal infections, etc The findings in pneumonia will be considered separately With respect to the other non-streptococcal diseases, the results have not indicated that any specific type of disorder is characterized by the presence of antifibrinolytic properties in the blood However, it is of interest to note that the average degree of antifibrinolysis in the control group of patients is somewhat greater than that found in normal persons For example, Myers, Keefer, and Holmes reported the average dissolution time of the two groups to be four and one-half hours and one hour, respectively Stuart-Harris inquired into the past history of the non-streptococcal cases which possessed antifibrinolytic properties, and in several instances noted that a preceding attack of tonsillitis or rheumatic fever may have accounted for the resistance to lysis However, even though occurrence of a concomitant or preceding hemolytic streptococcus infection may be responsible, in some instances, for the antifibrinolytic response of patients with non-streptococcal diseases, there is suggestive evidence that alteration in the blood associated with the acute active phase of infection may inactivate the fibrinolytic process In this connection the findings in pneumonia are of interest

Pneumonia Waaler (78) reported six cases of pneumonia,

in which the fibrin-clot was resistant In one of the cases the resistance persisted for two months Of five cases of pneumococcus pneumonia in adults reported by Tillett, Edwards, and Garner, the fibrin-clot of four was found to be susceptible both during the phase of acute, active disease and also during several weeks of convalescence In one patient, however, the blood obtained during active pneumonia exhibited maximum resistance, but within a few days after recovery there was a sudden and complete loss of resistance The rapid disappearance of the antifibrinolytic properties in this patient, associated with critical recovery, contrasted markedly with the gradual reduction over weeks or months of the resistance in patients with proven hemolytic streptococcus infections Stuart-Harris studied four cases of pneumococcus infection, two of which were pneumonia, one of mastoiditis, and one of pericarditis The fibrin-clot, in each instance, was found to be susceptible The tests apparently were performed during acute illness although no specific statements are made as to the time in the course of the illness at which the specimens of blood were obtained Myers, Keefer, and Holmes included cases of pneumonia in their large group of non-streptococcal diseases As previously mentioned, the average dissolution time of the whole control group was four and one-half hours The results with the blood from patients with pneumonia were not separated from the others

The conflicting results with pneumonia consist of the uniform finding by Waaler of high antifibrinolytic resistance, and the negative results of others, with the exception of the one case mentioned Waaler concluded from his studies of patients with bacterial endocarditis and pneumonia that the occurrence of antifibrinolytic properties in the blood of patients was not decisive evidence of hemolytic streptococcus infections

Interesting information is obtained from the studies of Lippard and Johnson concerning children with pneumonia They noted that, in the youngest patients, maximum resistance was present early in the disease but abruptly disappeared three to thirteen days after onset This finding was, however, not regularly obtained in all of the children with bronchopneumonia Boisvert

reported that in the pediatric age group, the majority of patients with pneumococcus pneumonia possessed antifibrinolytic resistance during the period of active disease but rapidly lost it after recovery

An interpretation of the data obtained in pneumonia is not apparent at the present time The factor of age of the patient may be important In addition, certain other possibilities warrant consideration The results obtained by some of the investigators were characterized by the fact that insusceptibility to fibrinolysis did not gradually appear over periods of time after the beginning of the infection, as occurs in usual immunological responses On the contrary, the high antifibrinolytic potency of early tests was followed by abrupt loss instead of gradual disappearance In view of this particular course of events, the possibility suggests itself that the inactivating effect exerted on the fibrinolysin of hemolytic streptococci by the blood of some cases of pneumonia is not dependent upon immunologically specific antibody but to non-specific substances present in the blood during acute illness and rapidly lost during recovery [1] On the basis that the fibrinolysin is an enzyme, it is interesting to speculate whether antienzymic effects comparable to the rise of antitrypsin which occurs during acute infection might account for the inactivation of the fibrinolytic enzyme An additional example of the effect of blood from cases of acute illness on hemolytic streptococci is furnished by the report of Tillett (70) who found that the serum of patients with pneumonia and other types of infection is highly streptococcidal, but the property is rapidly lost following cessation of active disease

On the basis of the present information, the interpretation of single tests may be summarized as follows

During active acute infections of streptococcal or non-streptococcal origin *In children,* antifibrinolytic properties are fre-

[1] In a recent personal communication Dr P L Boisvert of the Department of Pediatrics of Yale University School of Medicine outlined studies of antifibrinolytic immunity which are in progress It would be premature to comment in this article on his extensive but uncompleted data However, the findings, up to the present time, differentiate, in the pediatric age groups, between the specific immunity and probable non-specific inactivation

quently present (Lippard and Johnson, Boisvert) *In adults*, antifibrinolytic properties are frequently absent (66, 47, 63) but have been noted in pneumococcus pneumonia (78)

During convalescence, the development of antifibrinolytic properties, following non-streptococcal disease, has not been reported, following infections due to hemolytic streptococci, antifibrinolytic properties have appeared in approximately 60 to 80 per cent of the cases

The fact that the dissolution time in 10 to 15 per cent of normal persons is prolonged may account for the findings in which "moderate resistance" remains unchanged during acute illness and recovery Since "maximum resistance" has not been noted in normal healthy persons, its occurrence during convalescence is strong presumptive evidence of relatively recent infection due to a hemolytic streptococcus

Tillett and Garner reported that the serum from convalescent patients, the fibrin-clot of whose blood was resistant to dissolution, conferred antifibrinolytic properties when added to normal plasma Demonstration of the presence of antifibrinolysin in the serum suggested that specific resistance to fibrinolysis was not dependent upon properties of the fibrin substrate itself Van Deventer (75) isolated fibrinogen from the blood of several individuals Tests with the plasma-clot of these subjects indicated varying degrees of resistance However, when fibrinogen-thrombin preparations were used, no differences in susceptibility were noted The same author (75a, 76) tested twenty-eight commercial antistreptococcus sera by "passive transfer" to normal human fibrin He added the fibrinolysin in arbitrarily designated units to dilutions of sera and incubated the mixtures for 3 hours before adding to the fibrin constituents of the test Six of the sera were found to possess high titres of antifibrinolytic antibodies He added potent antisera to rabbit and monkey blood, allowed them to clot, and was able to demonstrate the antifibrinolysin in the serum expressed from the clots Van Deventer also attempted to immunize rabbits with fibrinolysin, using several cultural preparations as antigens However, the sera of the animals, even after many injections, failed to exhibit

antifibrinolytic properties when tested with susceptible human fibrin Schmidt (57) titrated samples of antistreptococcus horse sera, and according to the quantitative procedures, which he described, 0 0025 cc of highly potent sera were capable of inhibiting fibrinolysis

In some respects the use of serum in testing for antifibrinolytic resistance is more advantageous for quantitative titration than is plasma However, factors which have not up to the present time been studied in detail may condition the serological results Because of an insufficiency of experimental data it is unnecessary to discuss the problem in detail However, mention may be made of the fact that the thrombin contained in sera may be sufficiently high to coagulate, either wholly or partially, the substrate without the addition of $CaCl_2$ to oxalated plasma, or of specially prepared thrombin to fibrinogen Since both thrombin and antibodies are closely associated with the globulin fraction of blood, the possibility suggests itself that the thrombin of immune sera might carry antifibrinolysin into the forming fibrin, whereas the thrombin of normal sources results in the formation of susceptible fibrin It is apparent that the standardization of serological procedures must await additional studies

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